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Two Distinct Modes of Signaling by Vascular Endothelial Growth Factor C Guide Blood and Lymphatic Vessel Patterning in Zebrafish: A Dissertation

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TWO DISTINCT MODES OF SIGNALING BY VASCULAR ENDOTHELIAL GROWTH FACTOR C GUIDE BLOOD AND LYMPHATIC VESSEL PATTERNING IN ZEBRAFISH

A Dissertation Presented By

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Interdisciplinary Graduate Program

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DEDICATION

This thesis is dedicated to my parents Alphonse and Lillian Villefranc. I would not be where I am today without your sacrifice. Thank you.

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Where to start…

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ABSTRACT

Vascular Endothelial Growth Factor Receptor-3 (VEGFR3/Flt4) and its ligand Vegfc are necessary for development of both blood and lymphatic vasculature in vertebrates. In zebrafish, Vegfc/Flt4 signaling is essential for formation of arteries, veins, and lymphatic vessels. Interestingly, Flt4 appears to utilize distinct signaling pathways during the development of each of these vessels. To identify components of this pathway, we performed a transgenic haploid genetic screen in zebrafish that express EGFP under the control of a blood vessel specific promoter. As a result, we indentified a mutant allele of *vascular endothelial growth factor* c (*vegfc*), *vegfc*^{um18}. *vegfc*^{um18} mutants display defects in vein and lymphatic vessel development but normal segmental artery (SeA) formation. Characterization of this allele led to the finding that the primary defect in *vegfc*^{um18} mutants was a general failure in vein and lymphatic vessel sprouting. Further genetic and biochemical analysis of this mutant revealed profound paracrine defects, which likely result in the observed loss of lymphatic and venous structures. Furthermore, double mutant analysis demonstrated that defects during SeA formation in *vegfc*^{um18} mutants were masked by inputs from the Vegfa signaling pathway. Endothelial cell autonomous expression of *vegfc*^{um18} induced angiogenic effects on blood vessels while endothelial cells lacking *vegfc* displayed defects in tip cell occupancy, suggesting a cell autonomous-autocrine role for Vegfc during developmental angiogenesis. Finally, we present genetic evidence that links processing of Vegfc by Furin during the

formation of lymphatics in zebrafish. Together the data presented here suggest two discrete modes of signaling during blood and lymphatic vessel development, thus implying that regulation of Vegfc secretion and processing may play a pivotal role in the formation of these distinct vessel types in zebrafish.

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LIST OF USED ABBREVIATIONS

Abbreviation	Term
2A	Viral 2A peptide sequence
3’ UTR	Untranslated Region
ACV	Anterior Cardinal Vein
BP	Int, IHF
C-terminal	Carboxy-terminal
DA	Dorsal Aorta
DC	Duct of Cuvier
DLAV	Dorsal Longitudinal Anastomotic Vessel
dpf	Days post fertilization
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
ENU	Ethyl-N-nitrosurea
F	Filial
Flt4	Zebrafish ortholog of Vegfr3
GO terms	Gene Ontology Terms
hpf	Hours post fertilization
IHF	Integration Host Factor
Int	Intergrase
IRES	Internal Ribosomal Entry Sequence

Kdrl	Zebrafish ortholog of Vegfr2
LDA	Lateral Dorsal Aorta
LR	Int, IHF, and excisionase
LTBP	Latent TFG-β Binding Proteins
MO	Morpholino
nls	Nuclear Localization Signal
N-terminal	Amino-terminal
ORF	Open Reading Frame
p3E	3’ Entry clone, usually containing an epitope tag
p5E	5’ Entry clone, usually containing a promoter element
PC	Propeptide Convertase
PCV	Posterior Cardinal Vein
PHBC	Primordial Hindbrain Channel
PL	Parachordal Lymphangioblast
Plcg1	Phospholipase C gamma 1
PMBC	Primordial Midbrain Channel
pME	Middle Entry clone, usually encoding a gene of interest
Putant	Potential mutant
RTK	Receptor Tyrosine Kinase
SeA	Segmental Artery
SIV	Subintestinal vessels
SNP	Single Nucleotide Polymorphism

SS	Signal Sequence
SSLP	Single Sequence Length Polymorphism
TD	Thoracic Duct
Tg	Transgenic
TGFβ	Transforming Growth Factor-Beta
TGN	Trans Golgi Network
Vegf	Vascular endothelial growth factor
Vegfc	Vascular endothelial growth factor c
VEGF-CΔC	Human equivalent to Vegfc^{um18}
Vegfd	Vascular endothelial growth factor d
Vegfr2	Vascular endothelial growth factor receptor 2
Vegfr3	Vascular endothelial growth factor receptor 3
VHD	Vegf Homology domain
ZFYM	Zebrafish yolk membrane angiogenesis assay

CONTRIBUTIONS

ENU mutagenesis of Tg(*fli1a*:egfp)^{y1} males

Dr. Seong-Kyu Choe and Michael Kacergis; Sagerström Lab and Lawson Lab, respectively.

***In vitro* fertilization of oocytes**

Dr. Seong-Kyu Choe, Elizabeth Laver, Letitiah Etheridge and Michael Kacergis; Sagerström and Lawson Labs.

zfVegfc and zfVegfc^{um18} conditioned media

Dr. Michael Jeltsch. Alitalo Lab. Molecular/Cancer Biology Laboratory, University of Helsinki, fl

***vegfc* deficient mosaic analysis**

Dr. Stefania Nicoli. Lawson Lab

This rest of the work presented in this thesis was performed by Jacques A. Villefranc

CHAPTER I

INTRODUCTION AND LITERATURE SURVEY

The enclosed body plan of chordates requires a system to communicate with both the exterior and internal environment to permit efficient exchange of gases, as well as the transport of nutrients, liquids, and metabolic wastes. In vertebrates, these processes are carried out by the cardiovascular system, which include specialized tree-like tubular structures called blood and lymphatic vessels[1,2]. Blood vessels serve as a conduit for circulation of blood cells, exchange of gases, as well as the delivery of metabolic products to and from different tissues. By contrast, the lymphatic system consists of a network of blind-ended vessels that collect protein-rich fluid that exudes from blood vessels and return them back into blood circulation[3].

Blood and lymphatic vessel form through a process termed angiogenesis and lymphangiogenesis (see below), respectively [4-7]. During adulthood, proper functioning of both these vessels is essential for tissue homeostasis. Indeed, dysfunction of the cardiovascular system leads to a large number of pathologies including arteriovenous malformations, arteriosclerosis, stroke and lymphedema [8-10]. Additionally, blood and lymphatic vessels are fundamental during cancer progression and metastasis [11,12].

In this introduction I will describe: *1) Formation and signaling pathways responsible for blood and lymphatic development in zebrafish*. In particular, *2) Vascular endothelial growth factor c (Vegfc)/ Vegf receptor 3 (Flt4) signaling*. Although this pathway has been shown to be essential for both blood and lymphatic vessel development in vertebrates [8,13-18], how it orchestrates their

formation remains unclear. In order to dissect the mechanisms involved in this developmental process I performed a *3) forward genetic screen in a blood vessel transgenic zebrafish line to identify mutants that display loss of the primordial hind brain channel (PHBC), a defect associated with loss of* Vegfc/Flt4 *signaling.*

Descriptive overview of vascular development in *Danio rerio* (zebrafish).

Studying vascular development in vertebrates is often difficult due to internal embryonic development in some vertebrates, which precludes direct visualization of blood and lymphatic vessels. In recent years the zebrafish has emerged as an ideal model to study vascular development, due in large part to its external development, optical clarity and the existence of zebrafish transgenic lines that specifically label blood and lymphatic vessels with reporters encoded by fluorescent proteins driven by vessel specific promoters [19,20]. These attributes make the *in vivo* observation of vascular development relatively straightforward.

In this section I would like to introduce relevant aspects of blood and lymphatic vessel formation in the zebrafish, which will help define terms and processes that will be encountered in this thesis.

Blood vessel development in zebrafish

Blood vessel development is a highly conserved process in vertebrates that is governed by two major events: vasculogenesis and angiogenesis[4,21]. During vasculogenesis primitive mesodermal cells, called angioblasts, are specified to

become endothelial cells[21]. In the zebrafish, this process occurs between 10-20 hours post fertilization (hpf), when angioblasts migrate from the lateral plate mesoderm to the lateral line and form a primitive vascular cord of endothelial cells[22,23](Figure 1A-B). These cells then differentiate to form the dorsal aorta (DA) and posterior cardinal vein (PCV) following specific genetic programs [22]. In particular, differentiation of angioblasts into arteries requires inputs from the Notch signaling pathway [24]. More recently, studies in zebrafish found that Vascular endothelial growth factor (Vegf) controls the expression of Notch, which leads to arterial cell fate [25]. By contrast, recent studies have demonstrated that venous differentiation is modulated by COUP-TFII, an orphan nuclear receptor endogenously expressed in the vein, where it represses arterial gene expression[26,27].

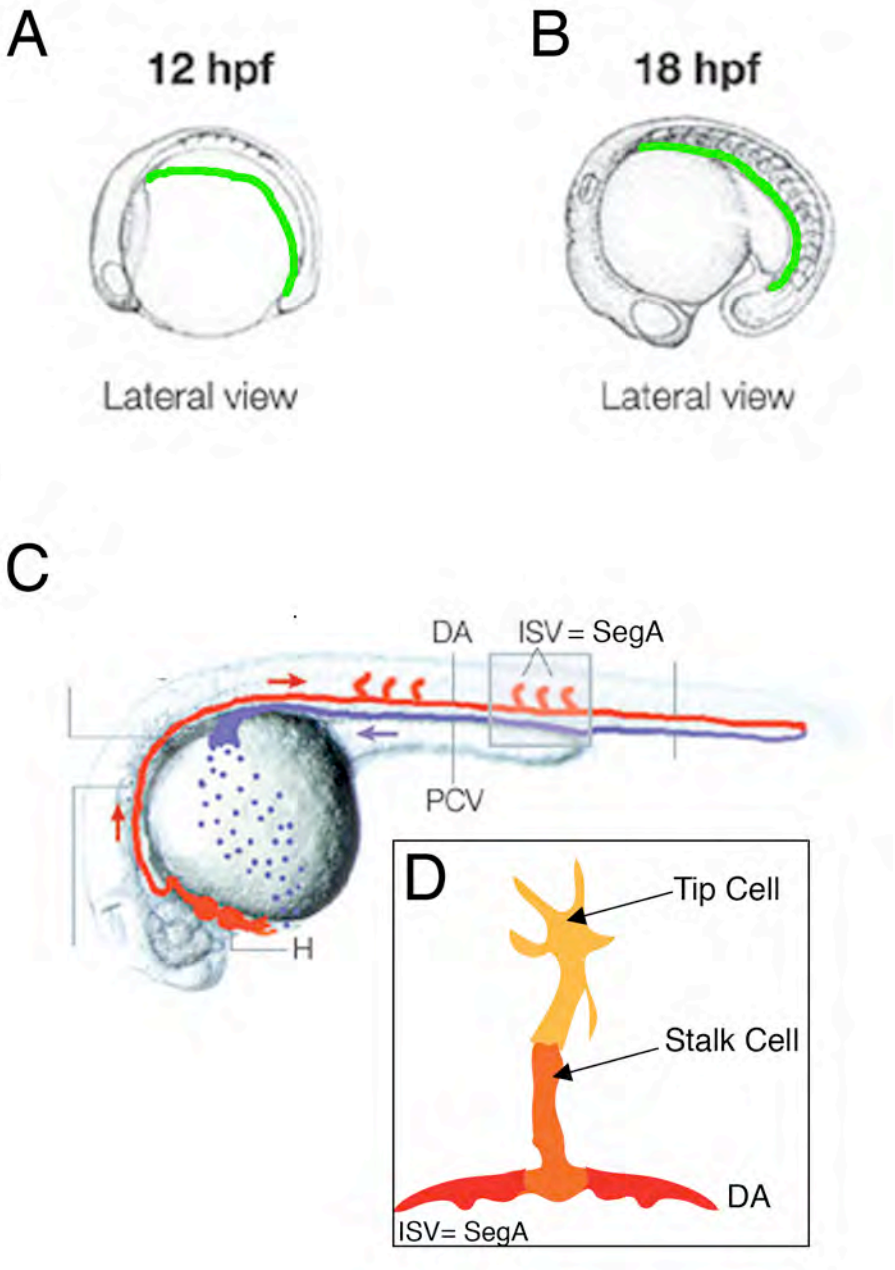

A
12 hpf
Lateral view
B
18 hpf
Lateral view
C
DA
ISV = SegA
PCV
H
D
Tip Cell
Stalk Cell
DA
ISV= SegA

Figure 1.I Vasculogenesis and angiogenesis in zebrafish

(A-C) 12,18, and 24 hour post fertilization (hpf) embryo, respectively. Anterior is to the left. Dorsal is up. (A-B) Migration of angioblasts from the lateral plate mesoderm to the lateral line. Green stripe represents angioblasts and eventual endothelial cells. (C-D) ISV=SegA, segmental artery. DA. Dorsal aorta. PCV Posterior Cardinal Vein. (C) Zebrafish undergoing SegA formation. (D) Sprouting SegA. Yellow cell denotes the tip cell. Orange cell denotes the stalk cell. Red cells denote the DA.

At 20 hpf, following vasculogenesis and arteriovenous differentiation, endothelial cells designated segmental arteries (SeA), begin to sprout from the DA and migrate dorsally in between somite boundaries (Figure 1C). This process is a classic example of angiogenesis, where endothelial cells are selected to sprout from preexisting vessels [7,28]. This sprouting requires coordination between two endothelial cell types, called tip and stalk cells (Figure 1 D). Tip cells acquire high proliferative and migratory behavior in order to lead the angiogenic sprout, while the stalk cell must lose these abilities in order to maintain their connection to the preexisting vasculature [29]. Studies in both mice and zebrafish have found that tip and stalk cell coordination during angiogenesis is governed in large part by the Vegf and Notch signaling pathways [28,30-32]. By 30 hpf, SeA reach the dorsal roof of somite boundaries where they appear to arrest an subsequently anastomose with adjacent SeA to form the dorsal longitudinal anastomotic vessel (DLAV), thus creating a primary DA derived vascular network[7].

At 36 hpf a new set of endothelial sprouts begin to emerge dorsally from the PCV and migrate in close proximity to the DA. During this process about half of these sprouts fuse with adjacent SegA in order to form secondary intersomitic vessels, which ultimately carry venous circulation. By contrast, studies have found that the remaining sprouts ultimately become lymphatic progenitors (Figure 2A, and below) [7,17,33,34].

Concomitant with trunk blood vessel formation, neurovasculature in the cranial region begins to arise from the anterior lateral mesoderm between 10-20 hpf.

One of the vessels formed in this region is the primordial hindbrain channel (PHBC), a bilateral primitive vein that returns cranial blood flow back to the heart via the ducts of Cuvier (DC) (Figure 2B, right panel). This vessel begins to form at 20 hpf, as endothelial cells from the primordial midbrain channels (PMBC) and anterior cardinal veins (ACV) begin to sprout in a bidirectional manner until they eventually fuse between 24-28 hpf [35](Figure 2B, left panel). Studies have demonstrated that

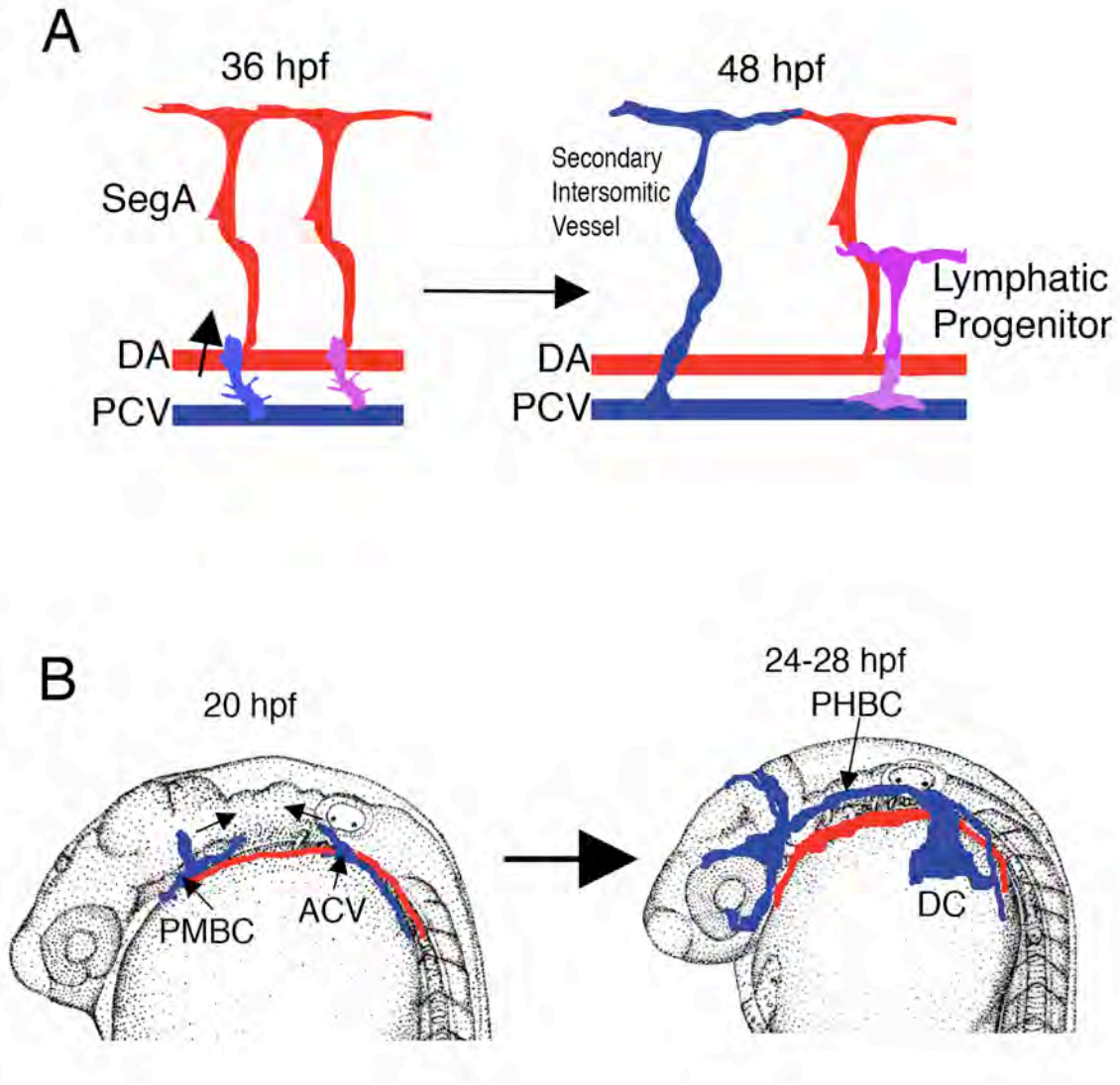

A
36 hpf
48 hpf
SegA
Secondary
Intersomitic
Vessel
Lymphatic
Progenitor
DA
PCV
DA
PCV
B
20 hpf
24-28 hpf
PHBC
PMBC
ACV
DC

Figure 2.I Secondary intersomitic vessel and Primordial Hindbrain Channel (PHBC) formation in zebrafish.

(A) 36-48 hpf embryo. Anterior is to the left. Dorsal is up. SegA. Segmental artery. DA. Dorsal aorta. PCV Posterior Cardinal Vein. Left Panel. Blue cell represents new set of endothelial cells sprouting dorsally from PCV. Purple cell represents endothelial cells sprouting dorsally from that will become lymphatic progenitors. Right panel. Represents the formation of secondary intersomic vessels and lymphatic progenitors. **(B)** 36-48 hpf embryo. Anterior is to the left. Dorsal is up. PMBC. Primordial Midbrian Channel (PMBC). Anterior Cardinal Vein (ACV). Primordial Hindbrain Channel (PHBC). Ducts of Cuvier (DC). Left panel. Represents formation of the PHBC beginning at 20 hpf. Black arrows represent bidirectional sprouting of the PHBC from the PMBC and ACV, respectively. Right panel. Complete PHBC between 24-28 hpf. Black arrow denotes PHBC.

formation this vessel is dependent on *vegfc*, a member of the Vegf family of signaling molecules and its receptor, *flt4* [14,15].

Lymphatic vessel development in zebrafish

Similar to blood vessel development, the formation of lymphatic vessels is a conserved process in vertebrates. Studies in mouse, zebrafish and frog have all demonstrated that lymphatic endothelial cells stem from the PCV [13,16,33]. In zebrafish, this process occurs between 1.5-2 days post fertilization (dpf) where endothelial cells, presumably lymphatic progenitors, begin to sprout dorsally from the PCV to horizonal myoseptum at which point they arrest and form parachordal lymphangioblasts (PL) (Figure 3A, 1.-2.). At 3 dpf, a subset of PLs migrate ventrally along SegA to the DA and PCV (Figure 3A, 3). Finally, between 4-5 dpf PLs extend laterally to form the future thoracic duct (TD), the first functional lymphatic structure homologous to that in mouse and humans [17,36] (Figure 3A, 4). Prior to sprouting, lymphatic progenitors are specified in the PCV [1,6](ref) by a genetic program that includes the transactivation of Prox1 by Sox18 [37,38]. To date, these two transcription factors are the only two markers of lymphatic vessel specification, however the mechanism involved in this process remains unclear[39]. Following specification, lymphatic endothelial cells sprout away from the PCV(above). Studies in vertebrates have demonstrated this process is dependent on Vegfc and its receptor Vegfr3 (Vegfr3 also Flt4) (see below)[16,33].

Cellular pathways involved in blood and lymphatic vessel development in vertebrates.

VegfA signaling. The Vascular endothelial growth factor (Vegf) family of signaling molecules plays a prominent role on the formation of both blood and lymphatic vessels in a largely non-redundant manner. One member of this pathway, VegfA, is the most important factor controlling blood vessel development in vertebrates(ref)[1,29]. VegfA is a growth factor that binds and induces the activation of its receptor Vegf receptor 2 (Vegfr2/ Kdrl in zebrafish), which in turn recruits various effector signaling molecules (discussed below) that result in the proliferation, migration and differentiation of endothelial cells [40]. Consistent with its role in these processes, vertebrates lacking *vegfa* display severe defects in blood vessel development. In particular, mice lacking at-least one copy of *vegfa* display severe defects in blood vessel differentiation and morphogenesis [41]. In zebrafish, genetic downregulation of *vegfa* negatively affects both differentiation and developmental angiogenesis [42]. Similar to loss of Vegfa, vertebrates lacking *vegfr2* display defects in endothelial cell differentiation and developmental angiogenesis [14,43-45].

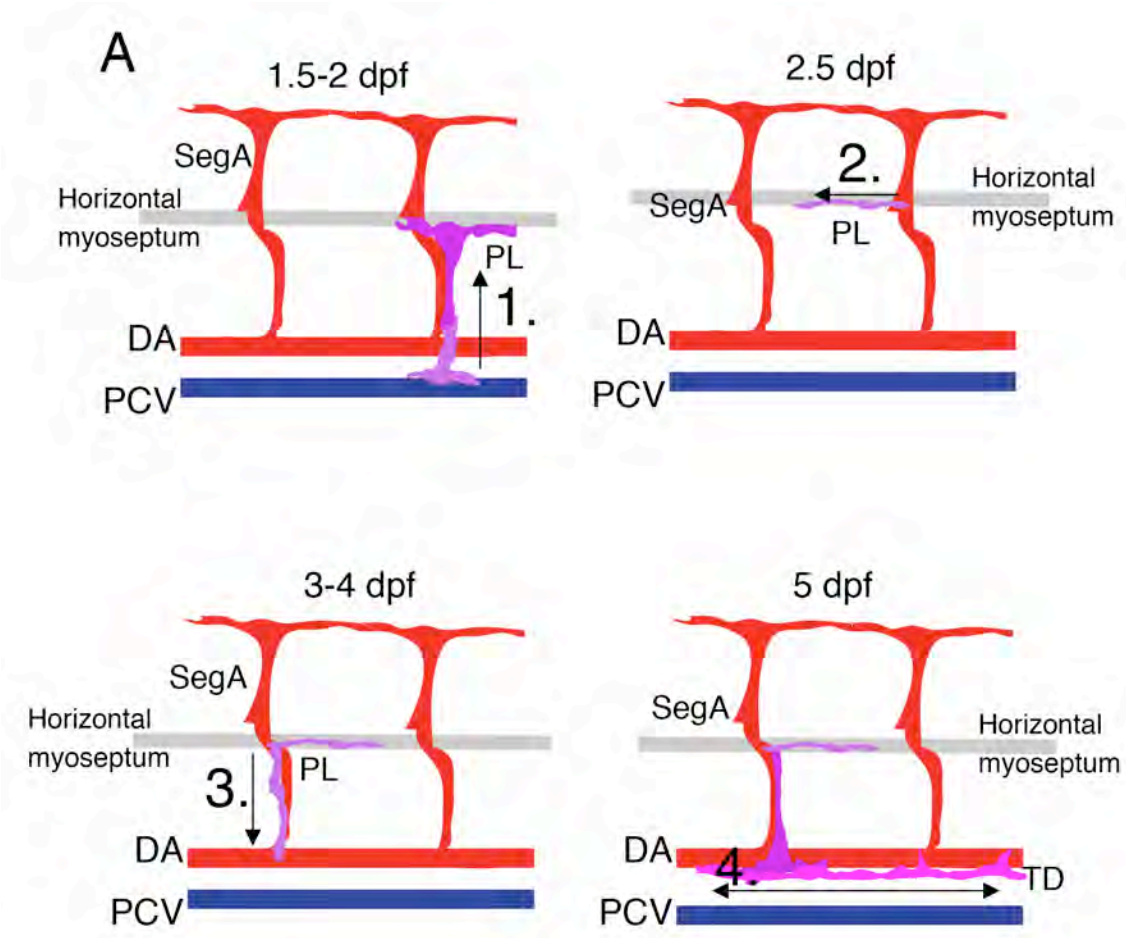


Figure 3.I Lymphatic vessel formation in zebrafish

(A) Schematic representing lymphatic vessel formation in zebrafish between 1.5- 5 dpf. Anterior is to the left. Dorsal is up. Ventral is down. SegA. Segmental artery. DA. Dorsal aorta. PCV Posterior Cardinal Vein. PL. Parachordal lymphatic progenitor. TD. Thoracic Duct. Horizontal myoseptum is denoted in grey. 1. Represents the dorsal sprouting of lymphatic progenitors to the horizontal myoseptum to form PLs at 1.5 dpf. 2. Represents arresting PLs at the horizontal myoseptum and their lateral migration at 2.5 dpf. 3. Represents ventral migration of PLs toward the DA and PCV at 3-4 dpf. 4. Represents the elongation of TD in between the DA and PCV at 5 dpf.

The diverse functions of VegfA during blood vessel development require tight control of its biological effects on endothelial cell function. One way in which VegfA regulates its effects on endothelial cells is through the expression of alternatively spliced variants of *vegfa*. These conserved isoforms of *vegfa* modulate ligand availability and thus control aspects of blood vessel development[1,46]. In particular, the expression of these isoforms differs in their capacity to bind heparin and thereby association with the extracellular matrix (ECM) . As a consequence, these distinct isoforms of VegfA vary in their mode of signaling. Soluble VegfA isoforms induce blood vessel enlargement where as ECM bound VegfA isoforms coordinate branching and migration of endothelial cells [9]. Paracrine VegfA induces blood vessel branching while autocrine VegfA induces blood vessel survival [9,47].

In addition to the distinct uses of ligand isoforms, VegfA signaling also coordinates its effects on blood vessels by differential usage of phosphorylated tyrosine residues within activated Vegfr2. In particular, the Y1175 residue of human Vegfr2 is required for its function, as mice containing a homologous mutation in this residue (Y1173F) display severe vascular and hematopoietic defects similar to *vegfr2* null mice [48,49]. This specific residue was shown to be required for its interaction with Phosholipase C gamma 1 (Plcg1) and the p85 subunit of PI3K [50,51]. Indeed, studies have demonstrated that the Y1175 is required for Plcg1-Erk1/2 and PI3K mediated proliferation of endothelial cells [50,51]. More recently, studies have found that PI3K-Akt signaling modulates Plcg1-

Erk1/2 signaling during various aspects Vegfa induced Vegfr2 signaling [52,53]. Additionally, Vegfr2 signaling is also regulated by its subcellular localization, adding another level of modulation to VegfA induced signaling [54].

While the aforementioned studies describe the activation of distinct intracellular signaling pathways in the modulation of Vegfr2 signaling, recent studies have also implicated the differential use of Vegfrs as a way to modulate Vegfr2 signaling during aspects of blood vessel development. In particular, studies have shown that Vegfr2 is able to heterodimerize with Vegfr3 and display distinct phosphorylation patterns as those induced by Vegfr2 and Vegfr3 homodimers [55]. More recently, studies have found that Vegfr2/Vegfr3 heterodimers are essential for angiogenic sprouting [56]. Taken together, these studies suggest that differential outputs of Vegfr2 signaling can be modulated by the activation of distinct intracellular signaling pathways, Vegfr2 localization or differential Vegfr usage.

Further insight into the functional interactions between members of the Vegf signaling pathway during blood vessel development has come from zebrafish studies performed in our lab [14]. We demonstrated that SegA formation is governed by *vegfa/kdrl/plcg1* signaling along with inputs from *vegfc/flt4* signaling (below). In particular, we showed that genetic down regulation of *vegfa*, chemical pan-inhibition of Vegfr signaling or a mutation in *plcg1* all resulted in the lack of SegA sprouting(ref). However, we found that a kinase dead allele of *kdrl* (otholog of Vegfr2) displayed less serve effects on the formation of SegA when compared

to pan-inhibition of Vegfrs, or separate deficiencies in *vegfa* or *plcg1* suggesting the involvement of another Vegfr(s) in the formation of SeA. Indeed, our studies demonstrated that genetic reduction of *flt4* or its ligand *vegfc* resulted in partial sprouting of SegA and zebrafish embryos deficient for both *flt4* and *kdrl* displayed a complete loss of SegA formation. These data suggest that cooperation between members of the Vegf signaling pathway is required during developmental angigenesis (Figure 4A). Interestingly, we found that defects resulting form deficiencies in either *vegfa* or *plcg1*, did not affect vein morphogenesis. However, we found that genetic reduction of either *flt4* or *vegfc* severely affected formation of head veins in particular the PHBC. More recently, others have demonstrated a requirement for *vegfc* and *flt4* during secondary intersomitic and lymphatic vessel formation [15,33,36](Figure 4B-C). Importantly, formation of these vessels were also independent of *vegfa* signaling[17,57](personal observation). As whole, these studies demonstrate that SeA formation is dependent on a pathway that largely involves *vegf/kdr/plcg1* with inputs from the *vegfc/flt4* signaling pathway. By contrast, formation of veins and the lymphatic vasculature appears to be independent of *vegfa/plcg1* and largely governed by the *vegfc/flt4* pathway implying that Flt4 signals through distinct downstream signaling molecules during vein and lymphatic vessel formation (Figure 4A-C).

Vegfc signaling Vegfc is a secreted growth factor and a member of the Vegf family of signaling molecules [40,58]. Vegfc functions by binding and inducing the

phosphorylation of its endothelial specific receptor Flt4, which in turn activates distinct intracellular kinases namely; Erk1/2, Akt, and JNK[59-61]. The activation of these downstream effectors is mediated by specific adaptor proteins that bind particular phosphorylated tyrosines within activated Flt4. Interestingly, the distinct use of these adaptor proteins at specific phosphorylation sites lead to separate biological outputs in endothelial cells[61]. In particular, Flt4 mediated cell survival is a consequence of direct binding of the adaptor protein CRKI/II to the phosphorylated Y1063 residue within Flt4, which in turn induces a signaling cascade that ultimately activates JNK. By contrast, Flt4 mediated cell proliferation and migration is the result of the direct recruitment of GRB2 to the phosphorylated Y1230 and Y1231 residues within activated Flt4, this leads to the activation of both Akt and Erk1/2. Although the differential activation of these signaling pathways lead to specific and separate biological outcomes in cells, the *in vivo* significance of this distinctive activation remains unclear.

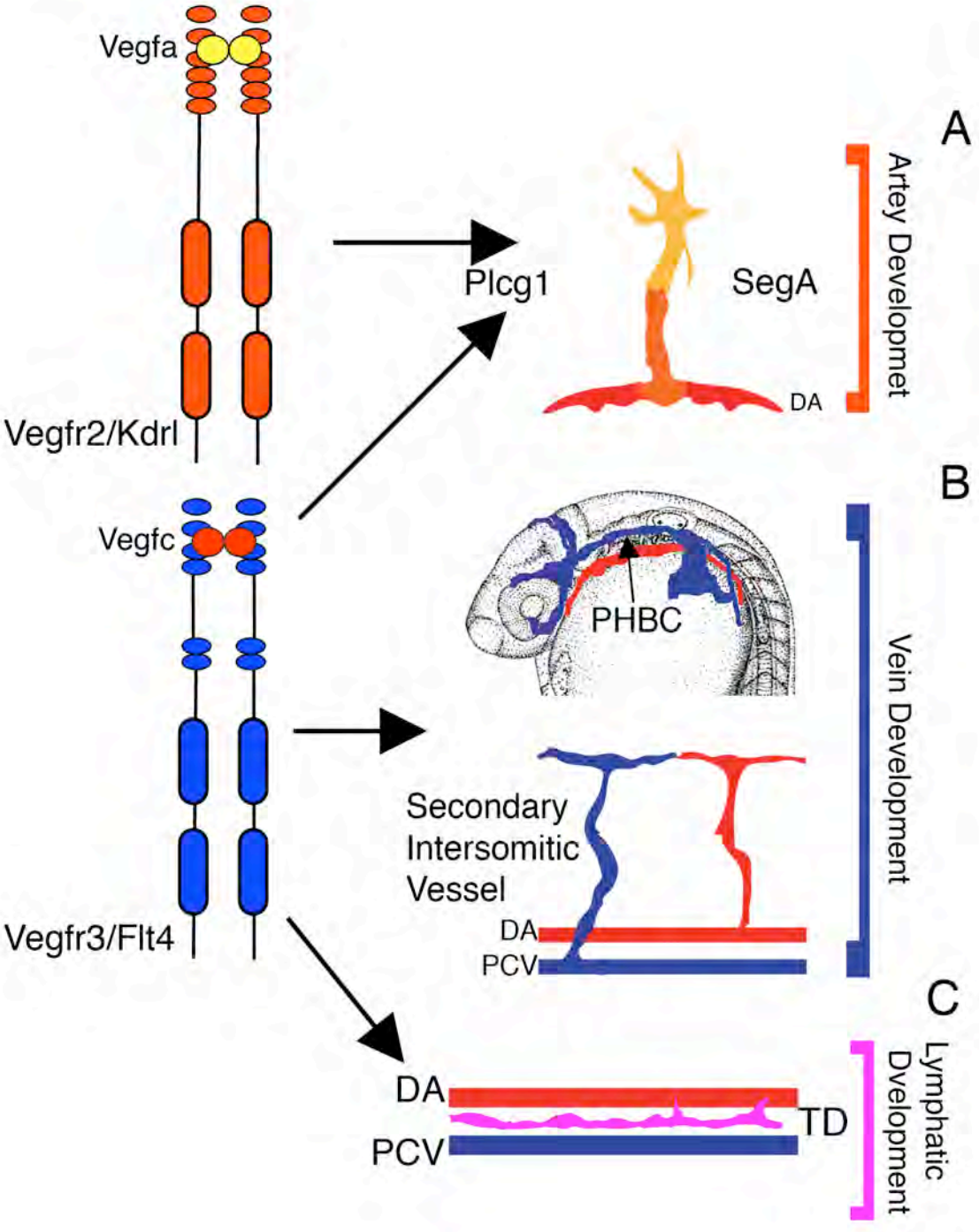

Vegfa
Vegfr2/Kdrl
Vegfc
Vegfr3/Flt4
Plcg1
A
SegA
DA
Artey Developmet
B
PHBC
Secondary
Intersomitic
Vessel
DA
PCV
Vein Development
C
DA
PCV
TD
Lymphatic
Dvelopment

Figure 4.I Requirement of Vegfc/Flt4 signaling during formation of distinct vessel types in zebrafish.

(A-C) SegA. Segmental artery. DA. Dorsal aorta. PCV Posterior Cardinal Vein. TD. Thoracic Duct. PHBC. Primordial Hindbrain Channel. (A) Artery development requires *vegf/kdr/plcg1* with inputs from the *vegfc/flt4* signaling pathway. (B) Vein development is largely governed by *vegfc/flt4* and independent of *vegfa/kdrl/plcg1*. (C) Lymphatic development is governed by the *vegfc/flt4* signaling pathway. This process is also independent of *vegfa/kdrl/plcg1* signaling.

During zebrafish development, *flt4* is concomitantly expressed in the axial vein and developing segmental arteries [14,28]. Consistent with its expression, *flt4* has been shown to be involved in the formation of SegA and secondary intersomitic vessels [14,15]. Accordingly, recent studies have shown its requirement during developmental and pathological angiogenesis in mice[62]. Flt4 signaling is also essential for lymphatic vessel development in vertebrates[8,15,63,64]. Indeed, humans with congenital missense mutations in Flt4 develop severe lymphedema. In most cases, these mutations eliminate the ability of Flt4 to undergo activation in response to Vegfc [8]. Similar to its receptor, Vegfc is involved in the formation of blood and lymphatic vessels in vertebrates [14,16,17,33]. In zebrafish, *vegfc* expression is restricted to the axial artery and developing SegA. Accordingly, downregulation of *vegfc* cause defects in SeA and vein morphogenesis (described above) [14]. In addition, mice and zebrafish lacking *vegfc* display defects in lymphatic progenitor sprouting, causing a complete loss of the lymphatic system[15,16,33].

Biosynthesis and structure of Vegfc. Unlike VegfA, Vegfc is synthesized a proprotein that undergoes a series of cleavages in order to be active. A similar synthesis of proproteins is also a hallmark of other growth factors such epidermal growth factor (EGF), transforming growth factor beta (TGFβ) and interleukin 1α and 1β [60,65]. An elegant biochemical study using human VEGF-C found that cleavage events define distinct domains within Vegfc, which include a Vegf

homology domain (VHD) required for receptor activation, an N-terminal domain and C-terminal silk homology domain of unknown function.

Upon biosynthesis Vegfc is folded into an anti-parallel dimer, which is linked by disulfide and non-covalent bonds (Figure 5A, 1.). Following homodimerization, Vegfc is cleaved at a conserved Furin cleavage motif that separates the VHD from the C-terminal domain (Figure 5A, 2.). The resulting secreted Vegfc is in the form of a tetramer, which undergoes further processing at the N-teriminus to remove the N-terminal domain to produce mature Vegfc, which is held together by non-covalent bonds (Figure 5A, 3.). The mature form of Vegfc is subsequently able to bind and activate both Vegfr2 and its major endothelial cell specific receptor Flt4 [58,60,66]. Interestingly, all forms of Vegfc are able bind and activate Flt4 at varying levels, but it is only the mature form that is able to bind and activate Vegfr2 [60].

Previous studies in cultured cells have suggested that the existence of the N-terminal and C-terminal domains flanking the VHD of Vegfc may regulate its activity during the various aspects of vascular development. This was borne out of the observation, that removal of the N-terminal domain and C-terminal homology domain of Vegfc induced activation of Vegfr2 and observable effects on blood vessel morphology, suggesting that these accessory domains may regulate its angiogenic and lymphangiogenic activity[60]. However, exactly how this proposed regulation occurs is unknown. More recently, studies have found that replacing the heparin-binding domain of $VegfA_{165}$, with the C-terminal domain of

VEGFC increased $VegfA_{165}$ angiogenic activity implying a role to the C-terminal domain in angiogenesis[67]. Inspection of C-terminal domain of Vegfc reveals strikingly similarity to the secretory silk protein (BR3P), which is characterized by series of repeated cysteine-rich motifs [59,60,66,68]. In addition to these motifs, the C-terminal domain of Vegfc also contains short motifs homologous to Epidermal growth factor(EGF)-like domains contained in other secreted proteins. These domains have been shown to facilitate protein secretion as well as association with the extracellular matrix (ECM) [69-71]. Interestingly, the structure of secreted Vegfc is similar to that of TGFβ; the C-terminal domain being similar to that of latent TGFβ-binding proteins, which are required for the association of TGFβ with the ECM and making it available to be active[72,73]. Taken together, these studies imply that the accessory domains of Vegfc may play a role in the regulation of Vegfc signaling. As a result, these domains may play a role in modulating aspects Vegfc activity during blood and lymphatic vessel development. However, to this date the exact biological function of these accessory domains remains unknown.

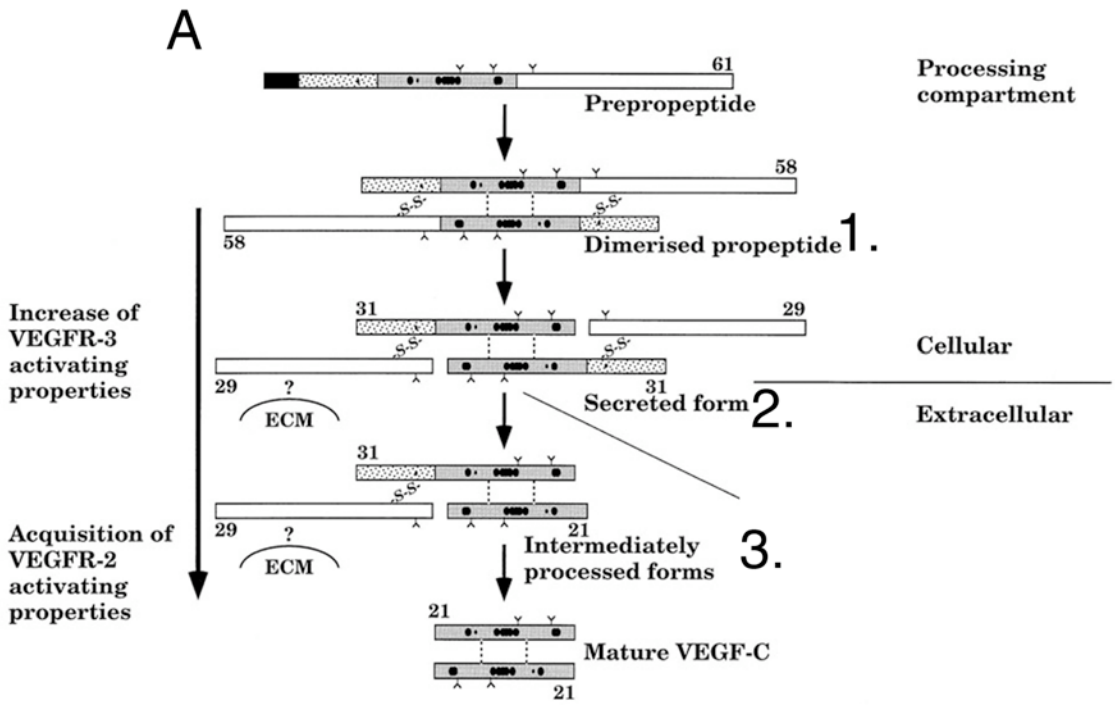


Figure 5.I Biosynthesis of Human Vegfc.

(A) Schematic model of the proteolytic processing of VEGF-C. The regions of VEGF-C polypeptide are marked as follows: signal sequence, black box; VEGF homology domain, grey box; N-terminal and C-terminal propeptides, dotted and open boxes, respectively. Cysteine residues and putative sites of N-linked glycosylation are shown as (Y); the cysteine residues in the C-terminal propeptide are not marked for clarity. Numbers indicate molecular mass (kDa) of the corresponding polypeptide in reducing conditions. Disulfide bonds are marked as -S-S-; non-covalent bonds as dotted lanes. The hypothetical binding of the C-terminal domain to the extracellular matrix (ECM). (1.) Denotes the dimerization of VEGF-C and. (2.) Represents cleavage of the C-terminal domain and the secreted VEGF-C tetramer. (3) Represents processing of the N-terminal domain to form mature VEGF-C. Importantly, this schematic represents processing of Human VEGF-C.

The role Vegfc processing during vascular development. Studies have found that processing at the Furin cleavage motif which separates the VHD from the C-terminal domain in Vegfc is required for its effects on blood vessel formation in cultured cells and fin regeneration in the adult zebrafish (Figure 5A, 1.) [74,75]. In particular, mutating this cleavage motif in Vegfc takes away its ability to induce blood vessel formation in cultured cells[74]. This study suggests that cleavage of Vegfc appears to have a functional role during blood vessel formation although its functional role during lymphatic vessel development remains unclear. This same study pointed to the requirement of the propeptide convertase (PC) Furin as a possible mediator of Vegfc processing at the C-terminal domain. In particular, the authors demonstrated that Furin directly processed Vegfc at the C-terminal domain of Vegfc in order to produce its mature form [74].

Furin function is required for the activation of various signaling molecules required for proper embryonic development[76]. Thus it is not surprising that mice lacking *furin* are embryonic lethal. As consequence, investigating its role during vascular development in mice would require the use of a tissue specific or conditional knock-out mice, which to date have not been reported. The zebrafish genome encodes to two *furin* genes (*furinA* and *furinB*), which have been shown to play partially redundant roles in craniofacial development [77]. In this study, the observation that a single mutation in *furinA* was not as severe as a deficiency for both *furinA* and *furinB* allowed for a more refined analysis of function during

embryonic development. This genetic particularity of *furin* in zebrafish may help shed light in to a possible role for *furin* during vascular development. To date studies accessing the role of either *furin* genes during vascular development in zebrafish have not been reported.

Modes of cell communication. Cells communicate using a variety of signaling molecules that regulate various aspects of cell biology including differentiation, migration, proliferation and survival. A subset signaling molecules called growth factors are peptides that interact with specific receptors on target cells. Binding of a ligand to its cognate receptor leads to receptor activation and subsequent initiation of signaling cascades that mainly lead to transcription of specific target genes, which in turn a regulate aspects of cell physiology.

The effects of growth factors on target cells depend on a variety of elements including; signaling contexts set by other growth factors, the differentiated state of the target cell, and the way in which the growth factor signals. Growth factors typically signal locally in either a paracrine or autocrine manner. In the case of the former, secreted growth factors from the signaling cell act as local mediators affecting target cells with cognate receptors that are in the immediate vicinity (Figure 6A). Most growth factors act in paracrine manner and they include: EGF, Insulin growth factor (IGF), Platelet-derived growth factor (PDGF), and members of the Vegf family growth factors among others. In particular, paracrine VegfA is required for chemotaxis and differentiation of angioblasts as well as endothelial cell proliferation[1,25,78]. Paracrine Vegfc is required for budding of lymphatic

endothelial cells from the cardinal vein to form the lymphatic system. Exogenous Vegfc and has also been shown to potently induce lymphangiogenesis [1,2,10,16].

In contrast to paracrine signaling, where the signaling cell and target are often different cell types, autocrine signaling is distinguished by the ability of cells to secrete growth factors that can signal back onto themselves (Figure 6B). Examples of this mode of signaling include: reinforcement of a developmental decision such as differentiation of a specific cell type, providing cells with information about a local environment, amplification and duration of an intracellular response, or persistent migratory behavior[79,80]. High levels of the cognate receptor often characterize cells displaying autocrine modes of signaling, while secretion of the autocrine ligand is tightly regulated. Examples of such factors include: Interleukin-2 (IL-2), TGF-β, EGF, as well as members of the Vegf family of growth factors[47,81-83]. In contrast to its paracrine role, autocrine VegfA promotes postnatal endothelial cell survival [47], while forced endothelial derived Vegfc has pro-angiogenic and lymphangiogenic effects on blood and lymphatic vessels[84].

More recently, studies have described a type of internal autocrine loop. This so-called intracrine mode of signaling requires an autocrine ligand and intracellular biologically active receptors in order to transduce signals (Figure 6C). The mechanisms governing intracrine signaling are largely unknown, although studies have found that intracrine signaling can occur at the level of the nuclear envelop, chromatin as well other subcellular compartments[85]. Studies have speculated

that intracrine mediated signaling result in self-sustaining signaling loops that behave as long lasting signals during aspects of cell physiology[85,86].

Together these context dependent modes of cellular communication result in the modulation of distinct aspects of cell biology.

Multiple roles for Vegfc/ Flt4 signaling in vascular development. It is clear that Vegfc/Flt4 is required for the formation of distinct vessel types that vary in location, architecture and function. However, the exact molecular mechanism(s) of how the formation of these vessel types is achieved remains unclear. A survey of the current literature suggests at least one possible mechanism may be the manipulation of varying downstream effectors in order to elicit discrete biological functions, which in turn may be regulated by homo-and heterodimeric Flt4 complexes. One other possibility may lie in the posttranslational processing and usage of accessory domains by Vegfc, which may in turn control its ability to signal during various aspects of vascular development. Together, these possible modes Vegfc/Flt4 modulation may contribute to the diverse biological effects of Flt4 signaling during vascular development. Thus, identifying players involved in Vegfc/Flt4 signaling could shed light on to how exactly this signaling pathway coordinates its diverse effects.

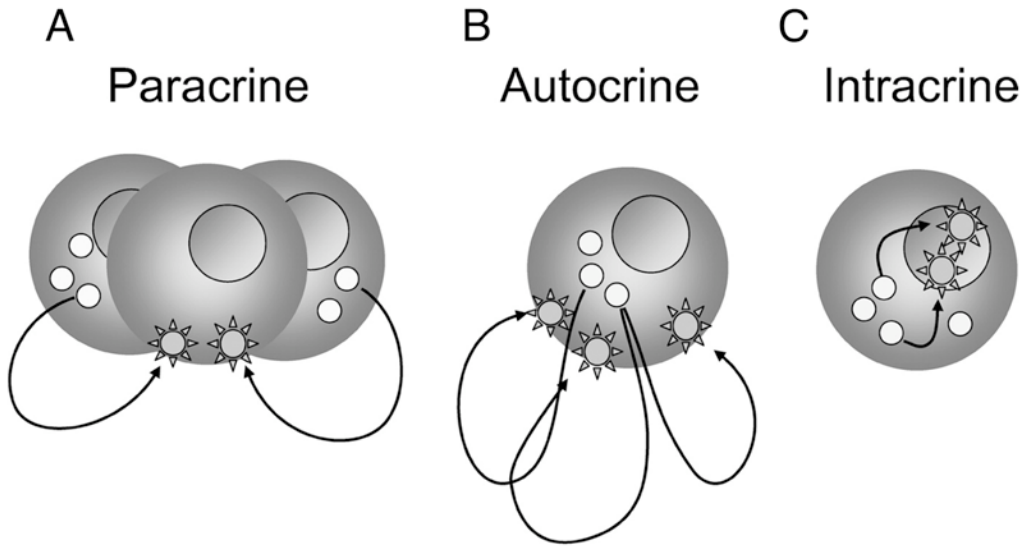


Figure 6.I Modes of growth factor signaling

(A-C) White circles represent a growth factor; sun-like graphics represent a cognate receptor. **(A)** Graphic depicting paracrine signaling. Growth factors secreted from the signal cell stimulate neighboring target cells expressing cognate receptors. **(B)** Graphic depicting autocrine signaling. A growth factor from the signaling cell signals back on its own receptor. **(C)** Graphic depicting intracrine signaling. Autocrine ligand and biologically active receptor induce an internal autocrine loop.

Forward genetic screens in zebrafish

Forward genetic screens have proved to be an efficient method to isolate genes involved in a biological process based on a desired phenotypic output. This forward genetic approach has been used in tractable genetic organisms such as *Caenorhabditis elegans* and *Drosophila melanogaster* to dissect complex pathways involved in various developmental processes [87,88]. In general, this method of screening affords an unbiased survey of the genes required for a developmental process. Making use of this approach in vertebrates requires the availability of an organism with rapid stereotypical embryonic development, optical clarity, and increased progeny size. In recent years the zebrafish has become an ideal system for the genetic analysis of vertebrate development due to the aforementioned characteristics. Initial mutagenesis studies in zebrafish relied on gamma irradiation in order to induce genetic lesions leading to embryonic mutant phenotypes. Although lesions affecting only a few base pairs were possible, the majority of induced mutations resulted in large deletions, making the resulting pleiotropic phenotypes more difficult to study [89,90]. As a result, the majority of mutagenic screens in zebrafish have been performed using N-Ethyl-N-nitrosourea (ENU)[45,91,92]. ENU is alkylating agent that has been shown to largely induce point mutations in pre-meiotic germ cells of the zebrafish. These point mutations can induce partial loss of function or null alleles[90].

In 1996 the Journal *Development* published a complete issue describing two large-scale mutagenesis screens in zebrafish; one performed in the Boston,MA

[93]and another in Tubigen, Germany [92], which identified mutants with obvious defects in early development that could be visualized under a simple dissection microscope. This screen for morphological defects in development helped identify mutants affecting a variety of developmental processes including; body axes determination, brain, blood and cardiovascular development, as well as mutations affecting adult phenotypes such as pigmentation patterns and fin development [91,92,94,95]. Although this approach was clearly able to identity mutants affecting nearly all aspects of development, more detailed studies of developmental processes and organ function lead to the initiation of screens focused on specific developmental processes. As result, researchers have employed more sensitive assays in order to identify mutants affecting a particular developmental process. These include, the use of histological sectioning, whole mount *in situ* hybridization and antibody staining as a way to identify mutants affecting a given aspect of development [96,97]. More recently, researchers have taken advantage of the genetic tractability of zebrafish in order to generate transgenic lines where *cis*-elements drive expression of a reporter a specific cell type, allowing serial observation of given developmental process and reducing the amount of downstream technical manipulations. Subsequently, researchers have gone back to the initial mutants identified by morphological defects and have used transgenic lines to perform secondary screens [20]. In some cases, others have used transgenic lines as a primary tool to screen for mutants affecting a given developmental process [45,98,99]. In addition to these methods of

screening, researchers have also devised screens in order to assess organ function and have identified mutants affecting lipid metabolism, cancer susceptibly and host response to infectious disease[98,100,101]. In most of these examples, general embryo morphology was overtly normal and would other wise be obviated in previous morphology based screening approach.

F3 breeding scheme. Most genetic screens in zebrafish (ex. Boston and Tubigen screens) are based on a three-generation crossing scheme. In this scheme, wild type parental males are treated with a given mutagen and crossed to wild type untreated females to produce an F1 family, where half of the progeny will harbor mutated chromosomes. The F1 families of fish (founder fish) are out-crossed to wild type fish to generate an F2 generation, half of which will contain mutated chromosome of interest. In turn, the F2 family of fish is randomly in-crossed to obtain recessive mutant offspring (Figure 7). F2 fish carry a myriad of mutations in their genome of which only a portion will become homozygous in a single in-cross. Therefore, many F2 crosses are required in order to recover as many mutants as is practical. As a result, this breeding scheme requires large amounts of space and personnel, which is cost prohibitive for a small research group.

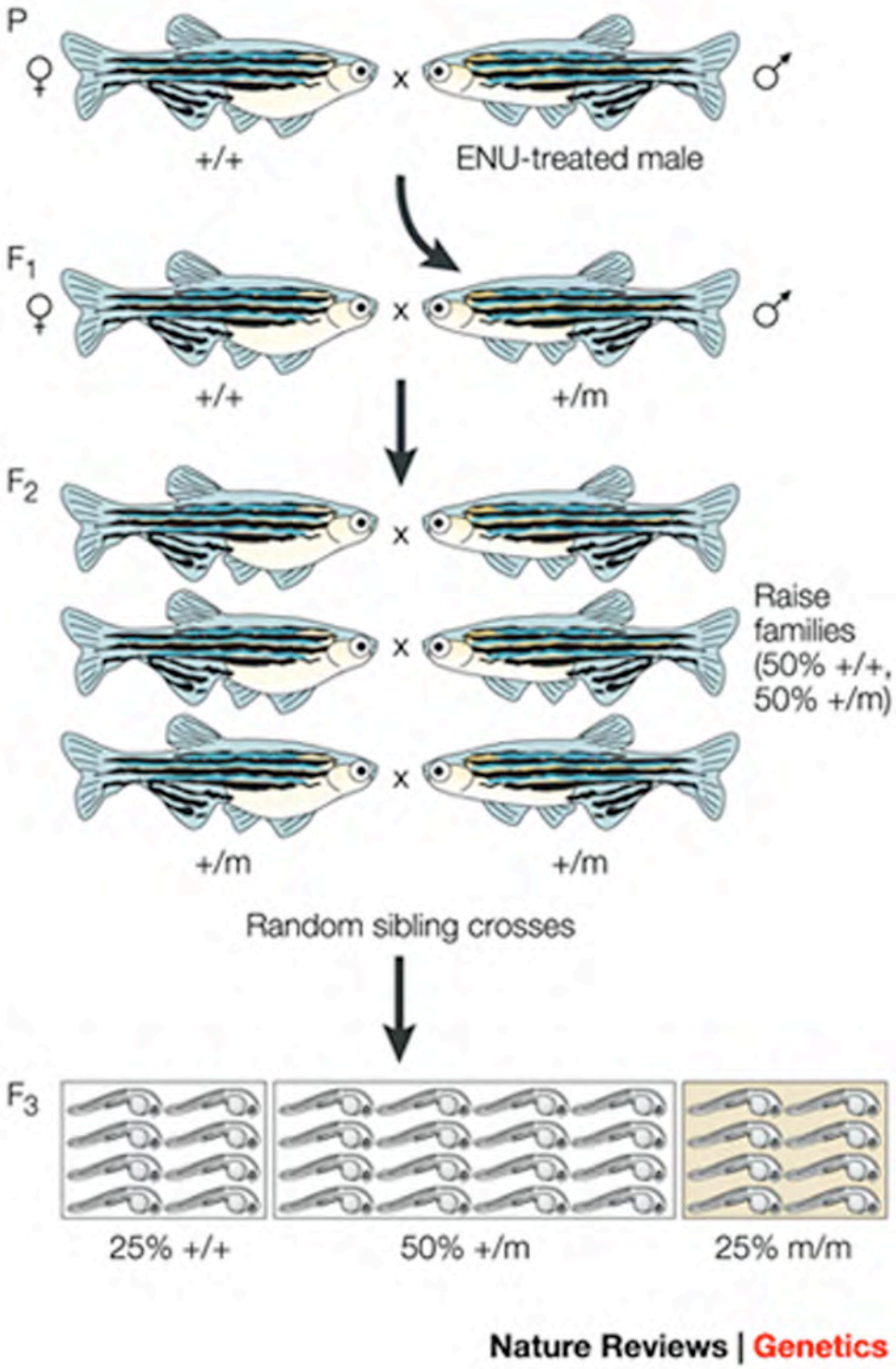

P
♀
x
♂
+/+
ENU-treated male
F1
♀
x
♂
+/+
+/m
F2
x
x
x
Raise
families
(50% +/+,
50% +/m)
+/m
+/m
Random sibling crosses
F3
25% +/+
50% +/m
25% m/m
Nature Reviews | Genetics

Figure 7.I Overview of F3 breeding scheme for ENU mutagenesis

In F3 screens, a mutagen, such as ethylnitrosourea (ENU), is used to generate hundreds of point mutations in the male pre-meiotic germ cells (spermatogonia). ENU-treated males are crossed to wild-type females to produce the F1 heterozygous progeny. F1 fish are then crossed to siblings to create F2 families, half of which are genotypically heterozygous for a specific mutation (m), whereas the other half are wild type. F2 siblings are crossed, and the resulting F3 progeny are 25% wild type (+/+), 50% heterozygous (+/m) and 25% homozygous (m/m) for a recessive mutation. Together, the Boston and Tübingen screens, starting from about 300 ENU founder males, involved raising more than 5,000 F2 families, analysing more than 6,000 mutagenized genomes and selecting more than 2,000 new developmental mutants for characterization.

Haploid breeding scheme. Recently, we along with others have taken advantage of the ability to generate haploids in zebrafish in order to perform mutagenesis screens [45,57]. In this breeding scheme, parental mutagenized males are out-crossed to wild type non-mutagenized females to generate an F1 family of females of which half harbor mutations in their chromosomes (Figure 8A). Haploid embryos are generated by *in vitro* fertilization of eggs generated from an F1 female and UV-irradiated sperm from a wild type zebrafish male. Since the DNA contained in the sperm has been irradiated it will not contribute to the offspring. Once haploids mutants are identified (Figure 8A), the F1 female is out-crossed to a wild type male zebrafish to produce an F2 family. This allows the introduction of polymorphic markers into the resulting progeny, which can then be used for genetic mapping of the causative mutation (Figure 8B). F2 siblings are then randomly in-crossed to obtain dipliod recessive mutant offspring (Figure 8B). The main advantage of this breeding scheme is that it reduces the amount of generations required in a screen by one. This is due to the fact that mutagenized DNA in a F1 female may represent only one allele at a given locus, therefore the establishment of haploid embryos allows the direct assessment of a recessive mutations.

One central drawback of haploid embryos is that they are inviable and die by four days post fertilization. Additionally, haploid embryos are mildly abnormal, exhibiting small cell size, abnormal brain development and short stature, which

often cause a crinkled trunk. Despite these defects, haploid embryos develop various structures in a normal manner, which allows for phenotypic screening of various developmental processes [90,102]. In particular, we have previously used this breeding scheme in a transgenic blood vessel background, in effort to indentify mutants affecting vascular development. We noted that although haploids often displayed morphological defects, blood vessel development was robust in wild type haploids thus making possible the identification of vascular mutants [45].

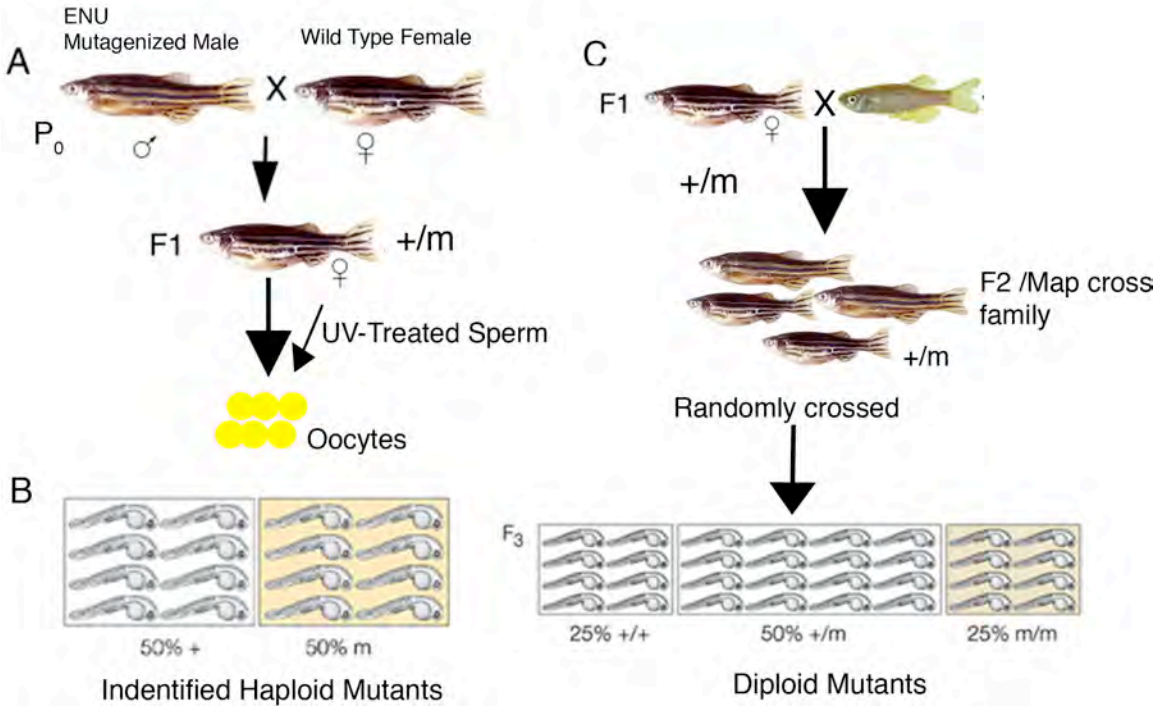


Figure 8.I Overview of Haploid breeding screen for ENU-based mutagenesis

(A) In haploid screens, a mutagen, such as ethylnitrosourea (ENU), is used to generate point mutations in the male pre-meiotic germ cells. ENU-treated males are crossed to wild-type females to produce the F1 heterozygous progeny. F1 females, which are genotypically heterozygous for a specific mutation (+/m), are squeezed for oocytes and fertilized with UV-treated sperm in order to generate haploid embryos. **(B)** A haploid clutch of embryos will contain 50% mutant and 50% wildtype. **(C)** The heterozygous mutant female (+/m) is subsequently out crossed to a wild type male in order to generate an F2/ Map cross generation. F2 siblings are randomly crossed to obtain dipliod recessive mutant offspring.

Isolation of causative mutation. Although the ENU method of mutagenesis has proven to be an efficient way to recover mutants affecting a given developmental process, the main drawback lies in the identification of the genetic lesion causing the observed phenotype. Inherent characteristics of the zebrafish itself along with enormous genomics efforts in the zebrafish community have helped alleviate the painstaking task of cloning mutations. First, zebrafish produce a very large clutch size, which allows the analysis of large number of meioses with which to map the mutated locus. Additionally, studies have found that the zebrafish and human chromosomes are surprisingly syntenic. Thus, once a few genes close to the mutation are identified, the syntenic region on the human chromosome can be surveyed to identify genes that display functional characteristics or expression patterns that may be linked to the observed mutant phenotype in zebrafish [103].

In recent years, work done in the zebrafish community has greatly facilitated the cloning of mutations recovered in forward mutagenic screens by enabling a series of readily available genomic resources. These include; a reliable physical and genetic linkage maps [104], along with the development of a large panel of known single sequence length polymorphisms (SSLPs) and the annotation of single nucleotide polymorphisms (SNPs) between strains. Together, these resources can be used as genetic markers for linkage analysis [90,102]. For example, once embryos are identified with a recessive phenotype, the large annotated panels of SSLPs can be used for initial bulk segregant mapping on wild type and mutant embryos to establish the genetic interval in which the

mutation lies. Fine mapping of the locus can further be achieved using SSLPs and known SNPs. Once a candidate gene is identified, it possible to take advantage of gene ontology (GO terms) as well as expression pattern and microarray data to further narrow down the list of candidate genes, which can be subsequently sequenced to identify the genetic lesion [102].

In some cases increased genomic complexity or misannotation of a given locus precludes the aforementioned approach from being enough to identify a given genetic lesion. As result, researchers have recently taken advantage of the wider availability of deep sequencing as way to facilitate the identification candidate genes. In one recent study, researchers employed deep sequencing methods to identify the causative mutation of the *magellan* allele. However, given the large size of the zebrafish genome, this first required the fine mapping of homozygous mutant versus heterozygous mutant embryos using SSLPs and SNPs [105]. As sequencing techniques continue move forward it conceivable that in the future zebrafish researchers will be able to directly sequence mutant progeny to identify causative mutations.

Goal of the project

Initial observations in our laboratory as well as those by others demonstrated that Vegfc/Flt4 signaling is involved in the formation of both blood and lymphatic vessels in vertebrates [13-15,33,62]. Studies have suggested that differential receptor usage and/or manipulation varying downstream effectors may be required to

elicit discrete biological functions [55,56,61,106]. Additionally, reports suggests that posttranslational processing and usage of accessory domains by Vegfc may control its ability to signal during various aspects of vascular development [60,67,74,75]. As a result, I decided to investigate how Vegfc/Flt4 signaling coordinates the formation of distinct vessel types during embryonic development in zebrafish. To do this I conducted a forward genetic screen in transgenic zebrafish embryos where blood vessel expressed Egfp under the control of an endothelial promoter, and screened for mutants that failed to form a PHBC; a defect associated with loss of Vegfc/Flt4 signaling.

Questions addressed in this thesis:

i. How does Vegfc/Flt4 signaling orchestrate the formation of distinct vessel types in zebrafish?

ii. Are there context dependent signaling mechanisms that regulate formation of distinct vessel types?

CHAPTER II

A TRANSGENIC HAPLOID SCREEN IN ZEBRAFISH TO IDENTIFY MEMBERS OF THE VEGFC/FLT4 SIGNALING PATHWAY.

Introduction

Danio rerio (Zebrafish) has become an ideal model system for genetic analysis of vertebrate development. This is due in large part to its *ex vivo* fertilization, optical clarity and genetic amenability. Forward genetic screens in other tractable genetic organisms such as *Caenorhabditis elegans* and *Drosophila melanogaster* have been used to uncover novel genes and dissect complex pathways involved in various developmental processes[87,88]. In general, forward genetic screens afford an unbiased survey of the genes required for a given developmental process. The majority of mutagenic screens in zebrafish have been performed using N-Ethyl-N-nitrosurea (ENU)[92,93]. Although ENU has proven to be an efficient mutagen, the central drawback lies in the subsequent identification of the causative mutation for a given observed phenotype.

Previously, a forward genetic screen to identify cardiovascular mutants relied on observed circulatory defects in mutant zebrafish embryos [95]. This screen identified mutants affecting cardiac contractility, such as the *silent heart* mutant, and mutants affecting circulatory function and vascular stability, such as *gridlock* and *bubble head,* respectively [107-109]. Although this method of screening yielded mutants with vascular defects, the presence of a normal circulatory loop is not always indicative of proper vascular morphology[14]. More recently, the use of transgenic lines, which specifically label zebrafish vasculature, has allowed the isolation of mutants affecting aspects of vascular

development in the presence of normal circulatory function. Examples of these mutants include *full of fluid* and *expando*, both of which affect lymphatic vessel formation, yet display normal circulation [15,34]. However, the three-generation crossing scheme (so-called F3 screen) used to identify these mutants is labor intensive and cost prohibitive for a small research group. (F3 screens are reviewed in Chapter I).

Recently, we and others have taken advantage of the ability to generate haploid zebrafish embryos and the availability of a blood vessel specific transgenic line in order to perform haploid mutagenesis screens for mutants affecting blood vessel development [45,57] (Haploid mutagenesis screens are reviewed in Chapter I). As a result, we identified mutants affecting various aspects of blood vessel development including endothelial cell specification, artery differentiation and developmental angiogenesis [45]. One drawback of the haploid approach is the inability to screen for phenotypes past 3 days post fertilization (dpf). Therefore, identifying mutants that affect aspects of vascular development following this time point, such as secondary vascular sprouting and lymphatic vessel formation, is difficult.

Studies in vertebrates have demonstrated that lymphatic endothelial cells originate from vascular endothelial progenitors in the vein[13,16,17,33]. Therefore, it is not surprising that these two cell types share common signaling pathways that govern their development. Among these are components of the Vascular endothelial growth factor (Vegf) family of signaling molecules. In particular,

previous studies have described the requirement of Vegfc signaling during both blood and lymphatic vessel formation in vertebrates[14,15,62]. Unlike other members of the Vegf family, Vegfc is expressed as a secreted proprotein that undergoes a series of proteolytic cleavage events that are required for its activation. The sites of these cleavage events define distinct domains within Vegfc, including a central Vegf homology domain, which is essential for receptor binding and a C-terminal silk homology domain of unknown function[60]. Vegfc functions by binding to and inducing phosphorylation of its cognate receptor tyrosine kinase, referred to as Vegfr-3, or Flt4 [58]. Flt4 in turn activates a number of different signaling effectors, including MAP kinases, JNK, and Akt. Interestingly, activation of each of these downstream targets can elicit discrete biological outputs in endothelial cells [61]. Consistent with the diverse roles of Vegfc/Flt4 signaling in cultured endothelial cells, we along with others have found that Vegfc/Flt4 signaling is required for cranial vein and segmental artery morphogenesis, as well as lymphatic vessel development in zebrafish [14,33]. Interestingly, defects in vein and lymphatic patterning appear to be independent of segmental artery defects [17,57]. Therefore, it is likely that Vegfc/Flt4 signaling acts in distinct signaling contexts throughout the formation of these distinct vessel types.

In this chapter, we employ a transgenic haploid screen to identify genes that are required for Vegfc/Flt4 signaling in distinct vascular contexts. We screen for haploid mutants that fail to form a primordial hindbrain channel (PHBC), a

defect known to be associated with loss of Vegfc/Flt4 signaling [14], and identify a mutant referred to as *um18*. This mutant displays defects in PHBC formation with a concomitant loss of lymphatic vessel patterning, although SeA formation is overtly normal. Positional cloning revealed that *um18* encodes a truncation allele of *vegfc* that eliminates its C-terminal domain. Further characterization of this mutant suggested that the observed lymphatic defects were due to earlier defects in the sprouting of lymphatic progenitors. Finally, we show that arterial expression of *vegfc* is sufficient to rescue lymphatic vessel patterning in *vegfc*^{um18} mutants. These findings show that it is possible to use a haploid transgenic screen as way to screen for and identify mutants involved in Vegfc/Flt4 signaling. Additionally, they suggest a paracrine role for Vegfc from the DA in the formation of the lymphatic system in zebrafish.

Materials and Methods

Zebrafish Handling and Maintenance

Zebrafish were housed and maintained in accordance to standard protocols described elsewhere.

Transgenic and Mutant Lines

The F1 Tg(*fli1a*:egfp)$^{y1\ m588.5}$ female was derived from a cross between ENU mutagenized Tg(*fli1a*:egfp)^{y1} male and a wild type Tg(*fli1a*:egfp)^{y1} female. The Tg(*fli1a*:egfp)^{y1} zebrafish line expresses Egfp in endothelial cells and is described elsewhere. The F_1 Tg(*fli1a*:egfp)$^{y1\ m588.5}$ was crossed to wild type TL (Tupfel Long fin) fish in order to obtain *um18*$^{+/-}$ carriers. ***um*** is the institutional line designation of the University of Massachusetts Medical School (ZFIN.org). To better visualize the TD we generated the Tg(*fliep*:dsRedEX)^{um13};Tg(*flk1*:crgfp)^{zn1} line. The Tg(*fliep*:dsRedEx)^{um13} line which labels both blood and lymphatic vessels was out crossed to the Tg(*flk1*:crgfp)^{zn1} line, which specifically labels blood vessels.

Mutagenesis and Screening

N-ethyl-N-nitrosourea (ENU) mutagenesis was performed as described previously [45,93]. The haploid screen was performed as follows. We treated homozygous Tg(*fli1a*:egfp)^{y1} male adult zebrafish with 3mM ENU for 1hr once

a week for a total of four weeks. After two weeks of recovery, Tg(*fli1a*:egfp)^{y1} ENU treated males were crossed to wild type females in order to clear unwanted mutagenized gametes which have been shown to harbor gross chromosomal aberrations [110]. Mutagenized Tg(*fli1a*:egfp)^{y1} males were subsequently crossed to untreated homozygous Tg(*fli1a*:egfp)^{y1} females to generate an F1 generation. At six months of age, F1 females were used to generate haploid embryos by *in vitro* fertilization. Briefly, squeezed eggs from F1 females were fertilized with UV irradiated sperm from wild type males. Each F1 female that successfully gave viable embryos was maintained in an individual tank. Tg(*fli1a*:egfp)^{y1} haploid embryos were subsequently screened at 30 hpf and at 50 hpf for defects in PHBC formation and SeA formation, as well as overall morphology. This was performed using a dissection scope equipped with epifluorescence. The screening took place once a week for a total of six months. F1 Tg(*fli1a*:egfp)^{y1} females that gave clutches in which 50% displayed a vascular phenotype were subsequently out-crossed to wild type male TL zebrafish to generate a map cross. Females generating haploid embryos that displayed general morphological defects were not used.

Positional Cloning of *um18*

To map the *um18* locus, the F1 putant (potential mutant) female was out crossed to a wild type TL male in order to generate an F2 generation map-cross. The resulting progeny from individual F2 in-crosses were assayed for

the formation of the PHBC at 26 hpf and circulation at 30 hpf. Finally, siblings were assayed for the formation of the TD at 5 dpf and again at 7 dpf. Embryos that failed to form both PHBC and TD were designated mutants and were subsequently arrayed in a 96 well plate, referred to as the 'mutant panel'. DNA was isolated, amplified, and assayed for polymorphisms as described elsewhere [57,111]. We performed candidate linkage mapping analysis for *vegfc* (ensembl ID ENSDARG00000069640) and *flt4* (ensembl gene ID ENSDARG00000015717). The *um18* mutation was mapped using simple sequence length polymorphisms (SSLPs). Before a specific SSLP marker was used to map the mutation, we first confirmed that it was polymorphic in F2 individuals that produced mutant embryos. The following primers were used to amplify specific SSLP markers: Z25069, 5' AGTTACTTGCGATCCGACCA 3' and 5' TCACTGGTAAACACCCCTACA 3'. Z11618, 5' TGAGTGGGTGTAGAGGGACA 3' and 5' GATAAGGGGCCTCTTGGTTC 3'. Z26040, 5' CCAGAGAACTCCACTTGTGC 3' and 5' CCCAACTCTGGTCACAATACAA 3'. Z24128, 5' CTCATCACCGCTGCAATAAA 3' and 5' ATCTTGTGGGAAACGAGTGG 3'. In addition, a single nucleotide polymorphism (SNP) was identified in the 3' UTR of *vegfc* was subsequently used for finer genetic mapping and genotyping; mutants linked to this SNP created an XcmI endonuclease restriction site. The *vegfc* 3'UTR locus was amplified using the 5' CCAACCAACAGTGCAGATG 3' and 5' GGTTCGAAATGAATGAGTTGC 3' primer pairs, and subsequently

digested with XcmI. Finally, phenotypically mutant and wild type embryos were separately digested in DNA lysis buffer. We then separately amplified all seven exons of *vegfc*, including about 100 bp of the surrounding introns and subsequently sequenced the CDS and all intron/exon boundaries of *vegfc*, in both phenotypically wild type and mutant embryos.

Phenotypic and Imaging analysis

Imaging of general morphology and circulation was performed using transmitted light on a Leica MZ FLIII using a Zeiss AxioCam MRc digital camera. Images of blood vessels and thoracic duct (TD) in Tg(*fli1a*:egfp)^{y1} embryos were obtained using a Leica DMIRE2 confocal microscope (Objective: HC PL APO 20x/ 0.70CS). To better visualize parachordal lymphangioblasts (PLs) and TD formation, microangiography was performed at 2, 3, 4, and 5 dpf using a 1% solution of rhodamine dextran in nuclease free water as described elsewhere [112]. Quantification of intersomitic arterial and venous connections was based on directional flow in 3 dpf embryos using light transmitted from a Leica MZ125 microscope. Segmental arteries and veins were counted laterally from the fifth somite through the end of the embryo, anterior to posterior.

***vegfc* rescue experiments**

To generate expression clones that allowed for tissue specific expression of *vegfc* or *vegfc*^{um18}, we generated Gateway Entry clones that encoded *vegfc*

and *vegfc*^{um18} using the following primers. pME*vegfc*: forward 5' GGGGACAAGTTTGTACAAAAAAGCAGGCTGGGCCACCATGCACTTATTTG GATTTTCTG 3' and reverse 5' GGGGACCACTTTGTACAAGAAAGCTGGGTTTTAGTCCAGTCTTCCCCAGT A 3' primers. To amplify generate pME*vegfc*^{um18} we used the forward 5' GGGGACAAGTTTGTACAAAAAAGCAGGCTGGGCCACCATGCACTTATTTG GATTTTCTG 3' and reverse, 5' GGGGACCACTTTGTACAAGAAAGCTGGGTCTTGTTTTGACAAACAGCTGC AGG 3' primers. In both cases we eliminated the stop codon from the primer and designed them so it would be in frame with a 3' epitope tag. To create a transgenic vector that allowed endothelial-specific expression of *vegfc,* *vegfc*^{um18}, or control *egfp,* we generated pTol2*fliebs*:vegfc-2Amcherry, pTol2*fliebs*:vegfc^{um18}-2Amcherry and pTol2*fliebs*:egfp-2Amcherry. To visualize expression, a viral 2A sequence was fused in frame with the middle entry ORF. The 2A sequence allows expression of multiple proteins from one transgene. We mixed pDestTol2pA, p5Efliebs, pMEvegfc w/o stp or pMEvegfc^{um18} w/o stp or pMEegfp3 with p3E2Amcherry at equal molar ratios and subjected them to a multisite LR reaction. Positive clones were identified through restriction enzyme digest analysis. To assay rescue of the TD formation in *vegfc*^{um18} mutants by either *vegfc* or *vegfc*^{um18} we injected the embryos from an in-cross of Tg(*fli1a*:egfp)^{y1};*vegfc*^{um18} heterozygous carriers with 25pg either of pTol2*fliebs*:vegfc-2Amcherry , pTol2*fliebs*:vegfc^{um18}-2Amcherry or

pTol2*fliebs*:egfp-2Amcherry. In all cases, embryos were concomitantly injected with 25pg of *tol2 transposase* mRNA to facilitate genome integration. Injected embryos were allowed to develop until 5 dpf and subsequently selected for embryos expressing mCherry in endothelial cells. mCherry expressing embryos were then scored for presence of the TD and ultimately genotyped.

MAZ-51 (VEGFR-3 inhibitor) Treatment

Tg(*flk1*:crgfp)^{zn1} ;Tg(*fliep*:dsRedEX)^{um13} were separately treated at 48 hpf, 72 hpf and 96 hpf with 10uM MAZ-51 in egg water and compared to 0.01% DMSO control cultured embryos. Embryos were treated and raised in 6-well tissue culture plates. The 10uM MAZ-51 solution was replaced daily. 48 hpf, 72 hpf and vehicle treated embryos were assayed for TD formation at 96 hpf. 96 hpf treated embryos where assayed for TD formation at 120 hpf.

Results

A transgenic haploid screen in zebrafish identifies a mutant that mimics loss of Vegfc/Flt4 signaling.

To cause mutations in the zebrafish pre-meiotic germline we treated homozygous adult male Tg(*fli1a*:egfp)^{y1} zebrafish with ENU (Figure 1A). Mutagenized males were subsequently out-crossed to wild type females Tg(*fli1a*:egfp)^{y1} to generate F1 families of females (Figure 1A) . We generated haploid embryos from F1 females (see methods) and subsequently screened for PHBC formation at 30 hpf, a defect associated with loss of Vegfc/Flt4 signaling (Figure 1B-C). Similar to previous haploid screens we noted that haploid embryos were morphologically short and stocky which contributed to kinks in the notochord and floor plate (Figure 1D). Additionally, we noted that morphogenesis of the brain often appeared to be abnormal, however eyes and otic vesicles formed normally in haploid embryos (Figure 1D). Despite these abnormalities cranial and trunk blood vessel morphology was usually normal in wild type Tg(*fli1a*:egfp)^{y1} haploid embryos (Figure 1E). In total we screened approximately 400 mutagenized genomes and found four females where approximately 50% of their haploid offspring failed to form a PHBC by 30 hpf (Figure 2A-D and Table 1). In addition to this PHBC mutant phenotype, we were able to identify two mutants that displayed ectopic formation of segmental arteries (Figure 2E-H and Table 1). A similar class of trunk vessel mutant has

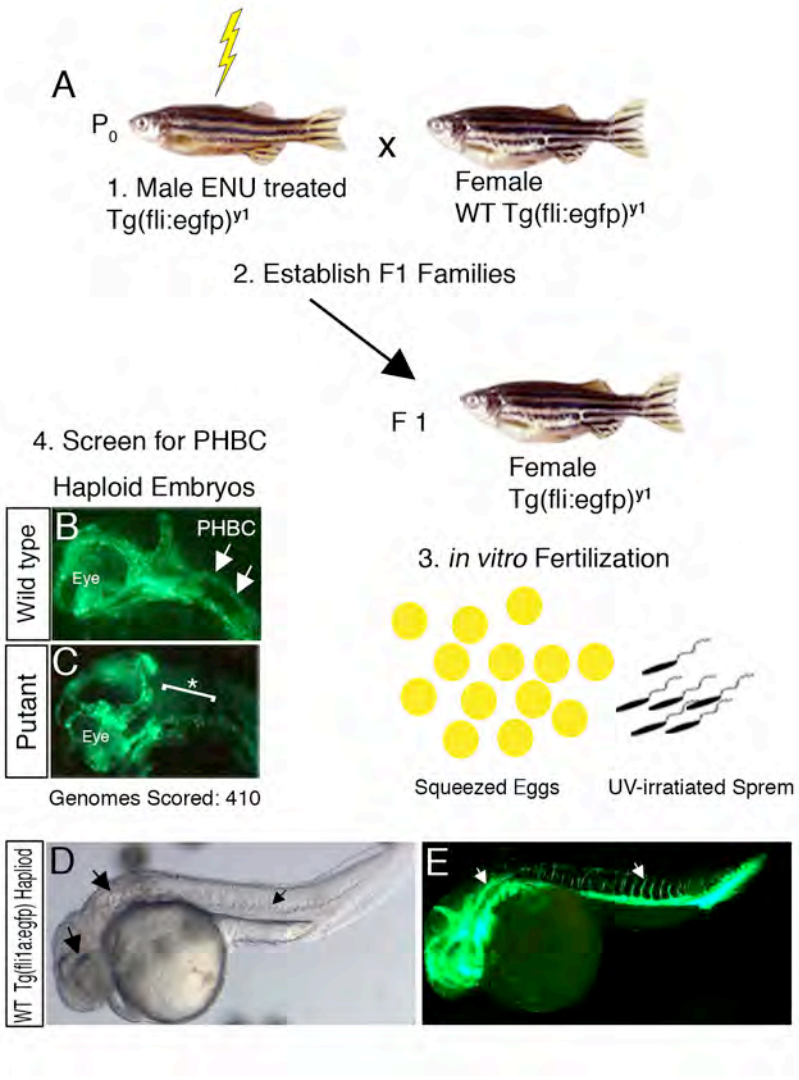

A
P0
X
1. Male ENU treated
Tg(fli:egfp)y1
Female
WT Tg(fli:egfp)y1
2. Establish F1 Families
F 1
Female
Tg(fli:egfp)y1
3. in vitro Fertilization
Squeezed Eggs
UV-irratiated Sprem
4. Screen for PHBC
Haploid Embryos
Wild type
Putant
B
PHBC
Eye
C
*
Eye
Genomes Scored: 410
WT Tg(fli1a:egfp) Haploid
D
E

Figure 1.II A Transgenic haploid screen in Tg(*fli1a:*egfp)^{y1} zebrafish to identify mutants affecting Vegfc/Flt4 signaling in zebrafish.

A. (1) P_0 Tg(*fli1a:*egfp)^{y1} males were treated with N-Ethyl-N-nitrosurea (ENU) to induce pre-meiotic germline. (2) Mutagenized males were subsequently out-crossed to wild type untreated Tg(*fli1a:*egfp)^{y1} females to establish F1 families. (3) Eggs from individual F1 females were *in vitro* fertilized with UV-irradiated sperm from wild type males. **B-C.** (4) Haploid embryos were allowed to develop and screened for the formation of a primordial hindbrain channel (PHBC) and segmental artery formation (SegA) at 28 hpf. B. White arrows indicate the presence of the PHBC. C. Brackets with an asterisk indicate the absence of a PHBC. Approximately 400 genomes were screened. **D-E.** Wild type haploid Tg(*fli1a:*egfp)^{y1} zebrafish embryo. D. Black arrows indicate normal eyes and otic vesicle. Black arrow also indicates a kink in the notochord. E. Illumination to visualize green fluorescence in haploid Tg(*fli1a:*egfp)^{y1} zebrafish embryo. White arrows indicate normal PBHC and SegA formation in the head and trunk, respectively.

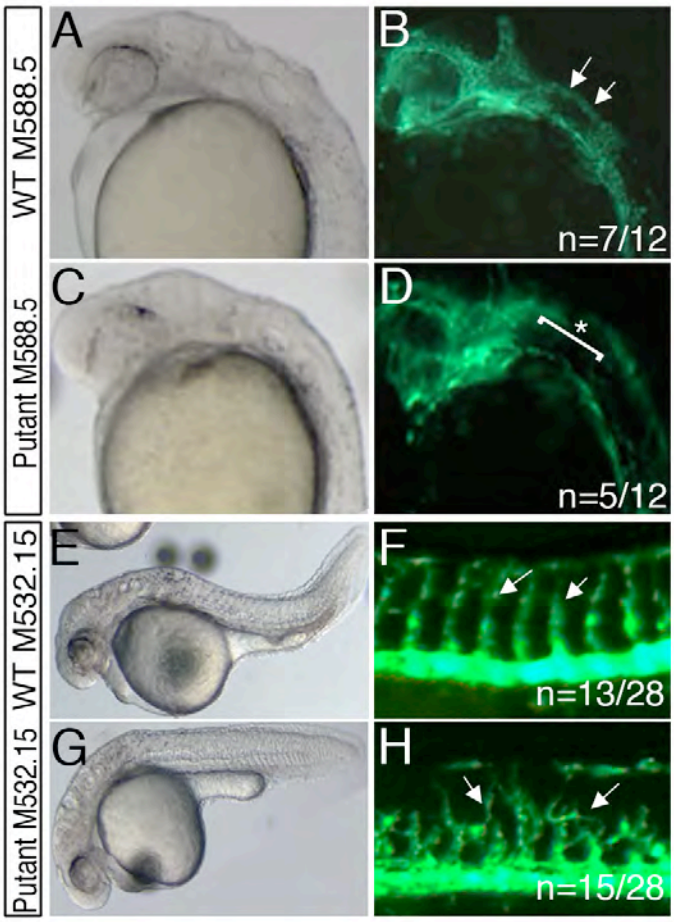

WT M588.5
Putant M588.5
WT M532.15
Putant M532.15
A
B
n=7/12
C
D
*
n=5/12
E
F
n=13/28
G
H
n=15/28

Figure 2. II Haploid mutants with defects in vascular development.

(A-F) Anterior is to the left. Dorsal is up. **(A-D)** Haploid progeny from F1 female (m588.5). A,C. Transmitted light illumination B,D. Illumination to visualize green fluorescence. (**A-B).** Wild type Tg(*fli1a:*egfp)^{y1} haploid embryo. White arrows indicate the presence of the PHBC. (n=7/12 embryos). (**C-D).** Mutant Tg(*fli1a:*egfp)^{y1} haploid embryo. D**.** Bracket with asterisk indicates absence of PHBC (n=5/12 embryos). **(E-H)** Haploid progeny from F1 female (m532.15). E,G. Transmitted light illumination F,H. Illumination to visualize green fluorescence. **(E-F).** m532.15 Wild type Tg(*fli1a:*egfp)^{y1} haploid embryo. F. White arrows indicate normal SeA formation. (n=13/28 embryos). (**G-H).** m532.15 mutant Tg(*fli1a:*egfp)^{y1} haploid embryo. H. White arrow indicate ectopic SeA formation.

Table 1

Haploid Phenotype	F1 Family (M)	Map Cross	F2 Family (M)	Gene	Allele	Mutation /Affected Domain
PHBC Deficient	552.4	Unable	ND	ND	ND	ND
	554.2	ND	ND	ND	ND	ND
	553.11	Unable	ND	ND	ND	ND
	588.5	Yes	910	*vegfc*	*um18*	Q→X/ C-Terminal
Ectopic Segmental Arteries	592.5	ND	ND	ND	ND	ND
	532.15	ND	ND	ND	ND	ND
Total	6	1	1			

Table 2

Phenotype	Scored wild type	Scored *um18*
PHBC + (30 hpf)	52	0
PHBC – (30 hpf)	0	13
TD + (5 dpf)	20	0
TD – (5 dpf)	0	45
		Total Embryos N= 65

Table 1.II List of putants identified in transgenic haploid screen.

ND- Not determined. Q- Glutamine (Gln). Primodial Hindbrain Channel (PHBC).

Table 2.II Primordial hindbrain channel (PHBC) and thoracic duct (TD) formation in progeny of *um18* heterozygous carriers.

Embryos were scored at 30 hpf. for the presence of the primordial hindbrain channel (PHBC) and subsequently scored at 5 dpf. for the presence of a thoracic duct (TD). N= total amount of embryos scored.

been identified previously and appears to be linked to the *plexinD1* gene [45,113]. Once we identified PHBC "putants" (potential mutants); each F1 female, presumed heterozygous carries of the mutated allele, was individually crossed to wild type male TL zebrafish. This served two important purposes, the first being confirmation of the vascular defect observed in the haploid embryos in diploid progeny. Second, it allowed the introduction of polymorphic markers that were used for linkage analysis (see below). Of the four putant F1 females, we were only able to establish one viable F2 family, where the subsequent F3 progeny displayed identical loss of the PHBC, similar to mutant haploid embryos (Table 1). Together, these results suggest that we are able to identify a mutant phenotype that phenocopies loss of Vegfc/Flt4 signaling in zebrafish using a haploid transgenic screening method. In addition, these results further confirm the use of the haploid transgenic screen as a way to rapidly identify vascular mutants in zebrafish.

A truncation allele of *vegfc* perturbs vein and lymphatic development.

In-crossing individual heterozygous *Tg(fli1a:egfp)*^{y1} F2 family members (derived from putant F1 females) yielded F3 progeny where about 20% of embryos failed to form a PHBC at 30hpf; these mutants were otherwise morphologically indistinguishable from wild type embryos with a fully formed PHBC (Figure 3A-D Table 2). The lower than expected ratio of PHBC mutant progeny suggested this defect was partially penetrant. In fact, we noted that PBHC formation recovered by 40 hpf in affected embryos (personal

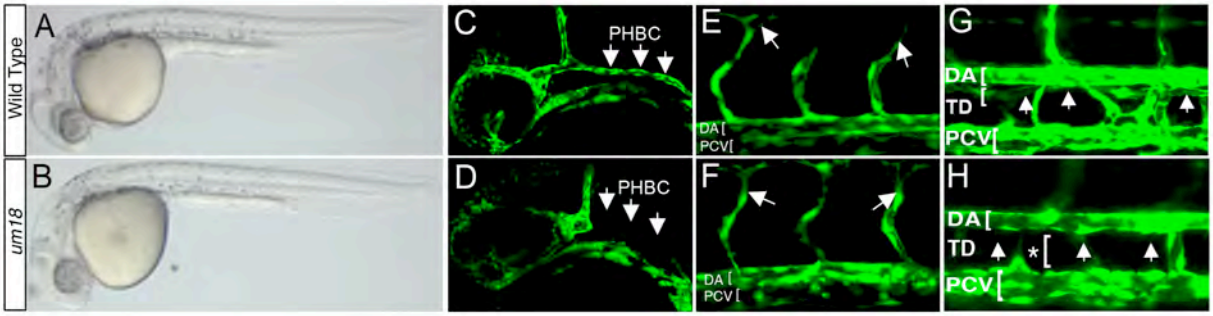

Wild Type
um18
A
B
C
D
E
F
G
H
PHBC
PHBC
DA
PCV
DA
PCV
DA
TD
PCV
DA
TD
PCV

Figure 3.II *um18* mutants display defect in vein and lymphatic vessel development.

(A-H) Anterior is to the left and dorsal is up. DA, Dorsal Aorta. PCV, Pisterior Cardinal Vein. PHBC. Primordial Hindbrain Channel. TD. Thoracic Duct. **(A-B)** Transmitted light illumination. A. Wild type *um18* diploid Tg(*fli1a:*egfp)^{y1}. B. Mutant *um18* diploid Tg(*fli1a:*egfp)^{y1} embryo. **(C-D)** Confocal micrographs of head blood vessels at 30 hpf. C. PHBC of *um18* wild type diploid Tg(*fli1a:*egfp)^{y1}. White arrows denote PHBC. D. PHBC of a mutant *um18* diploid Tg(*fli1a:*egfp)^{y1} embryo. Arrows indicate absence of PHBC. **(E-F)** Confocal micrographs of trunk blood vessels at 30 hpf. E. Segmental arteries of wild type *um18* diploid Tg(*fli1a:*egfp)^{y1}. Arrows indicate normal segmental arteries. F. Segmental arteries of mutant *um18* diploid Tg(*fli1a:*egfp)^{y1} embryo. Arrows indicate normal segmental arteries. **(G-H)** Confocal micrographs of trunk blood vessels at 5 dpf. G. TD in wild type *um18* diploid Tg(*fli1a:*egfp)^{y1} embryos. Arrows indicate formation of the TD. Brackets denote its location with respect to the DA and PCV. H. TD in mutant *um18* diploid Tg(*fli1a:*egfp)^{y1}. Arrows and bracket with asterisk indicate absence of the TD.

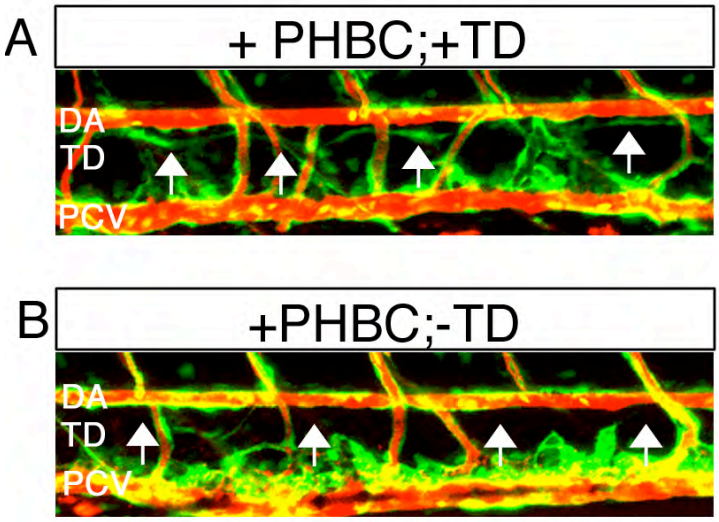


Figure 4.II Lack of thoracic duct formation in embryos that form a normal primordial hindbrain channel.

(A-B) Confocal micrographs of trunk blood vessels at 5 dpf. in progeny from a *um18* carrier in-cross. Embryos were subjected to Microangiography (Red). A. Embryo from *um18* carrier in-cross with normal formation of PHBC (+) and TD (+). White arrows indicate presence of the TD. B. Embryo from *um18* carrier in-cross with normal formation of PHBC (+) and absence of TD (-). White arrows indicate absence of the TD.

observation). To further investigate the extent of defects in this mutant, we examined other aspects of blood development and formation lymphatic vessels. Examination of SeA formation, which takes place between 22-30 hpf in wild type embryos (Figure3E), revealed that SeA were overtly normal in embryos that failed to form a PHBC (Figure 3F). We next investigated whether formation of the lymphatic system was affected in mutant embryos. In wild type *Tg(fli1a:egfp)*^{y1} zebrafish, the first identifiable lymphatic vessel is the thoracic duct (TD), a thin blind-ended vessel that normally forms by 5 dpf and lies between the dorsal aorta (DA) and posterior caudal vein (PCV; Figure 3G). By contrast, we observed that the TD was absent at 5 dpf in mutants that failed to form a PHBC. Additionally, we also noted that a high proportion of embryos failed to form a TD (70%) within that same clutch, suggesting that this mutation caused haploinsuffiency during TD formation (Figure 4A-C and Table 2). However, we detected carriers at expected frequencies in adults suggesting that they are capable of recovering from this early defect (personal observation). We subsequently designated this mutant allele, *um18*.

To map the *um18* mutant allele we performed candidate gene mapping analysis which involves four steps: *I) Identification of mutant carriers and establishment of the mutant phenotype (above). 2) Identification of polymorphic markers in the vicinity of the candidate genes;* followed by *3) analysis of linkage between a polymorphic marker and the observed mutant phenotype.* Linkage is based on the analysis of recombination events (meioses) between

an inherited marker and mutant allele causing the observed phenotype. If the phenotype was not linked to a polymorphic marker then you would expect to observe independent assortment of the marker and the phenotype. However, if the phenotype was linked to a polymorphic marker then you would expect to observe co-segregation of the marker with the mutated locus due to less recombination events, which suggest the phenotype is “linked” to a given marker. Once a linkage interval is established, one can proceed with the sequencing of candidates to identify the causative genetic lesion.

Since the phenotypic defects observed in *um18* mutants were similar to Vegfc/Flt4 loss of function [14,15], we treated them as potential genes harboring the mutation (candidate gene) and tested whether *um18* was linked to either loci. We identified single sequence length polymorphisms (SSLPs), also known as CA markers, which were in the vicinity of *flt4* and *vegfc*. We subsequently assessed linkage to the given locus by analyzing the number of recombination events in a panel of phenotypically mutant embryos. We observed that *um18* mutants displayed random assortment of polymorphic CA makers that were in close proximity to the *flt4* locus (personal observation). We next investigated whether *um18* mutants were linked to polymorphic CA markers adjacent to the *vegfc* locus. Indeed, we found that *um18* lies within 0.2cM (1cM= 0.01 recombination frequency) of CA marker z11618 of chromosome 1 (Figure 5A). Finer mapping of the locus was accomplished by identifying a single nucleotide polymorphism (SNP) in the *vegfc* 3’UTR.

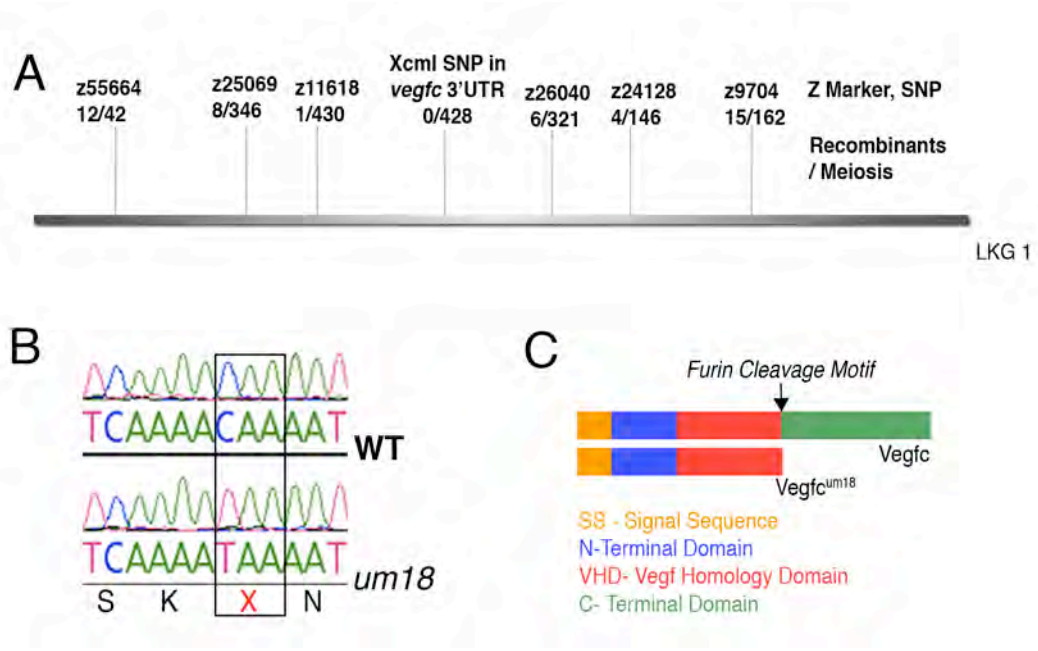


Figure 5.II *um*^{18} mutation is a truncation allele of Vascular endothelial growth factor c.

A. Schematic representing region linked to *um18* in chromosome 1. Polymorphic markers used in this study are indicated above the map. Ratios represent the number of recombination events in mutant embryos over total meioses analyzed.

B. Grey outline box indicating the CAA (Gln) → TAA (X) transition in the fourth exon of the *vegfc* coding sequence. **C.** Graphic describing Vegfc protein domains. SS-Signal Sequence(Orange). N-Terminal domain(Blue). VHD-VEGF Homology Domain(Red). C-Terminal Domain(Green). Black arrow represents a Furin cleavage motif.

We observed zero recombination events in 428 analyzed meioses indicating tight linkage of *um18* to the *vegfc* gene (Figure 5A). Subsequent sequencing of the *vegfc* coding sequence in *um18* mutants revealed a CAA (Gln) to TAA (Ochre) transition (Figure 5B). The resulting truncating allele of *vegfc* (*vegfc*^{um18}) eliminates the C-terminal domain of Vegfc, which is separated from the Vegf homology domain (VHD) by a Furin cleavage motif (Figure 5C).

To confirm that the loss of thoracic duct in *vegfc*^{um18} mutants was indeed due to loss of *vegfc*, we performed rescue experiments. Since *vegfc* is normally expressed in arterial endothelial cells, we drove expression of either wild type *vegfc* or *vegfc*^{um18} in arterial endothelial cells of *vegfc*^{um18} mutant embryos using an Ets-binding element from the *fli1a* gene (Also used in Appendix I). To visualize ligand-expressing cells, both *vegfc* and *vegfc*^{um18} were fused in frame to *mcherry* separated by a viral 2A peptide sequence, which allows the production of multiple proteins from a single transgene. In control *vegfc*^{um18} mutant embryos expressing *fliebs:egfp-2Amcherry* in arterial endothelial cells, we noted full penetrance of *vegfc*^{um18} lymphatic defects (Figure 6A-C and J), while transient mosaic expression of *fliebs:vegfc-2Amcherry* rescued TD formation in greater than 60 percent of mutant embryos (Figure 6D-F and J). This rescue was mosaic and proximal to arterial cells displaying red fluorescence, sites of presumed *vegfc* transgenic expression (Figure 6E-F, yellow arrowheads). By contrast, arterial cells expressing *fliebs:vegfc*^{um18}-

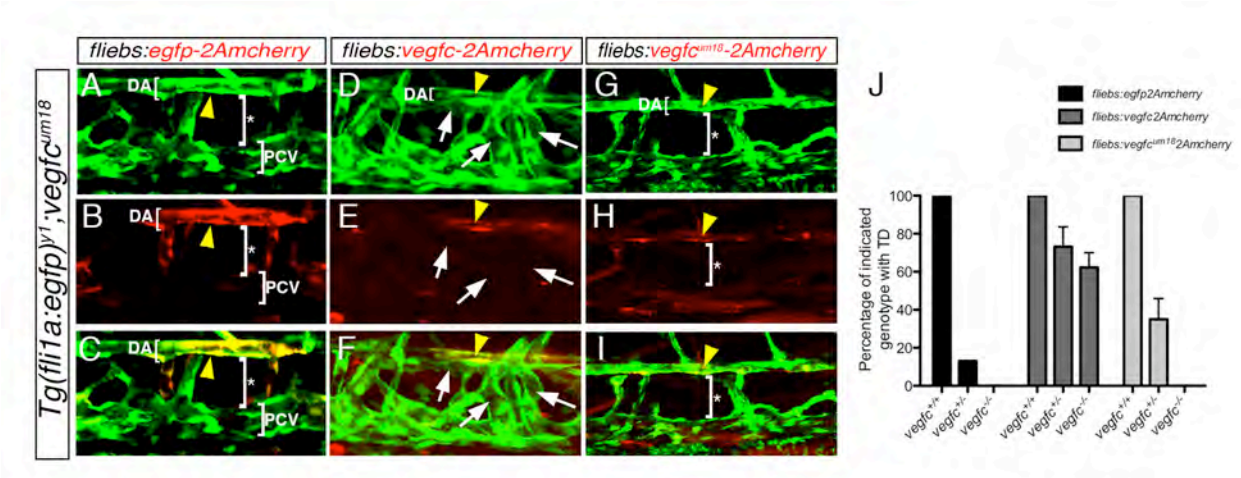


Figure 6.II Arterial expression of transgenic *vegfc* rescues lymphatic development in *vegfc*^{um18} mutants.

(A-I) Confocal micrographs of trunk blood vessels at 5 dpf. Anterior is to the left dorsal is up. DA, Dorsal aorta. PCV. Posterior Cardinal Vein. TD. Thoracic Duct. **(A-C)** Tg*(fli1a:egfp)*^{y1}*vegfc*^{um18} mutant injected with 25pg of Tol2 transposase and pTol2egfp-2Amcherry. Yellow arrowheads indicate colocalization of Egfp and mCherry. Bracket with asterisk indicate absence of TD. **(D-F)** Tg(*fli1a:egfp)*^{y1}*vegfc*^{um18} mutant injected with 25pg of Tol2 transposase and pTol2vegfc-2Amcherry. Yellow arrowheads indicate colocalization of Egfp and mCherry. White arrows indicate mosaic rescue of the TD. **(G-I)** Tg*(fli1a:egfp)*^{y1}*vegfc*^{um18} mutant injected with 25pg of Tol2 transposase and pTol2vegfc^{um18}-2Amcherry. Yellow arrowheads indicate colocalization of Egfp and mCherry. Bracket with asterisk indicate absence of TD. **(J)** Quantification of rescue. Expressed as the percent of indicated genotype of an injected clutch of *um18* heterozygous carriers.

2Amcherry failed to rescue TD formation in *vegfc*^{um18} mutant embryos (Figure 6G-J). Taken together, these data demonstrate a requirement for the C-terminal domain of Vegfc during vein and lymphatic vessel development. Furthermore, they suggest that arteries guide lymphatic patterning, in part, by providing a source of *vegfc*.

vegfc^{um18} mutants display general failure in vein and lymphatic progenitor sprouting.

To investigate why *vegfc*^{um18} mutants fail to form lymphatics we analyzed various aspects of vascular development that are required in order to form lymphatics in zebrafish. Recently, a descriptive analysis of lymphatic development in zebrafish found that similar to mice; lymphatic vasculature in zebrafish is derived from endothelial cells that sprout from the PCV. Following initial sprouting, these venous cells either form secondary intersomitic veins or **(1.)** migrate to the horizontal myoseptum where they become parachordal lymphangioblasts (PLs) by 1.5 to 2 dpf. **(2.)** PLs then migrate dorsally or ventrally, where they **(3.)** eventually become part of the TD between 3-5 dpf (Figure 7A). To determine what step in this process was affected in *vegfc*^{um18} mutants, we scored for the formation of PLs and secondary intersomitic veins in embryos from an in-cross of heterozygous carriers. While PLs were evident in the horizontal myoseptum of wild type siblings (Figure 7B white arrows, E), they were completely absent in homozygous mutants as well as *vegfc*^{um18} heterozygotes (Figure 7C-E, brackets). We next investigated secondary

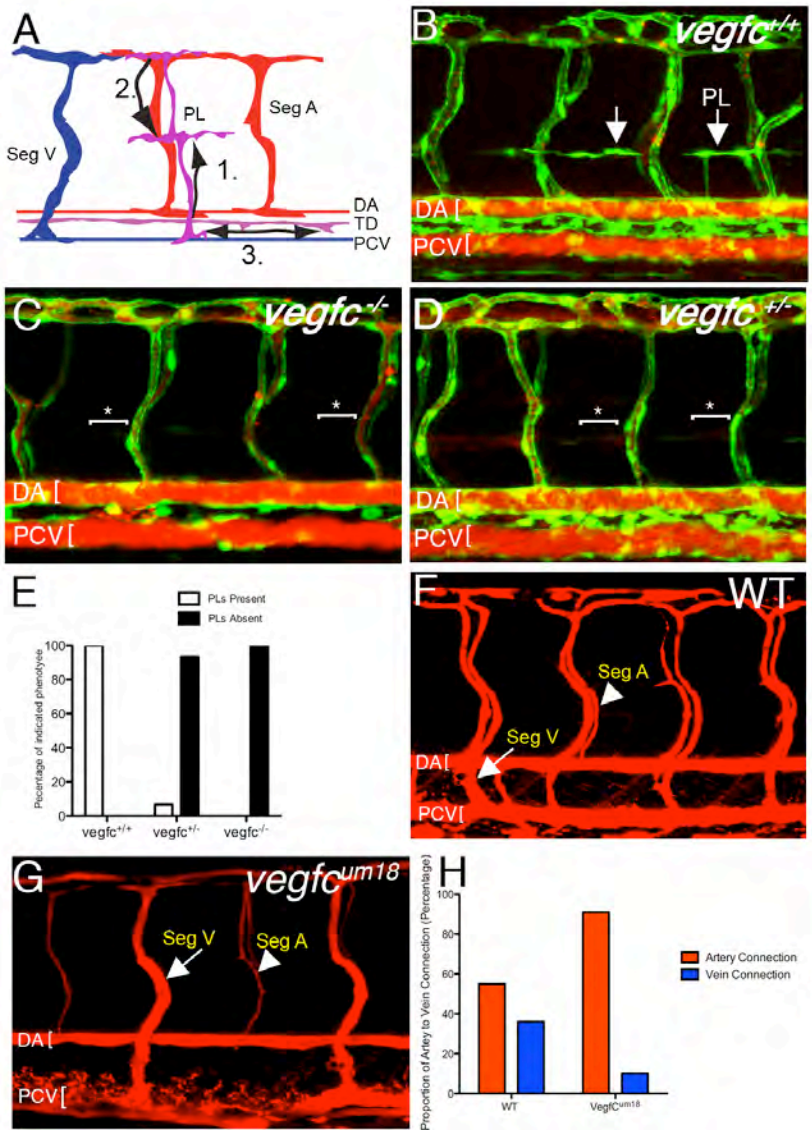

A
2.
PL
Seg A
Seg V
1.
DA
TD
PCV
3.
B
vegfc+/+
PL
DA
PCV
C
vegfc-/-
DA
PCV
D
vegfc+/-
DA
PCV
E
PLs Present
PLs Absent
Percentage of indicated phenotype
100
80
60
40
20
0
vegfc+/+
vegfc+/-
vegfc-/-
F
WT
Seg A
Seg V
DA
PCV
G
vegfcum18
Seg V
Seg A
DA
PCV
H
Proportion of Artery to Vein Connection (Percentage)
100
80
60
40
20
0
WT
VegfCum18
Artery Connection
Vein Connection

Figure 7.II *Vegfc^{um18}* mutants display primary defects in lymphatic progenitor and vein sprouting.

(A) Diagram describing lymphatic vessel formation in zebrafish. 1. Dorsal sprouting of parachordal lymphangioblsts (PLs) from the posterior cadinal vein (PCV) 1.5-2dpf. 2-3. Ventral migration of PLs to form part of the TD. **(B-D)** Confocal micrographs of trunk blood vessels at 2 dpf. Anterior is to the left dorsal is up. DA, Dorsal aorta. PCV. Posterior Cardinal Vein. Parachoradal lymphangioblast (PL). Embryos were subjected to microangiography. B. Tg*(fli1a:egfp)^{y1}vegfc^{um18}* homozygous wild type embryo. White arrows indicate presence of PLs. C. Tg*(fli1a:egfp)^{y1}vegfc^{um18}* homozygous mutant embryo. White brackets with asterisk indicate absence of PLs. D. Tg*(fli1a:egfp)^{y1}vegfc^{um18}* heterozygous mutant embryo. White brackets with asterisk indicate absence of PLs. **E.** Quantification of PL formation given the indicated genotype. Embryos where scored for the presence or absence of PLs at 2dpf. and subsequently genotyped. Values are shown as percent embryos of indicated genotype displaying either presence or absence of PLs. **(F-G)** Confocal micrographs of trunk blood vessels at 3 dpf. Anterior is to the left dorsal is up. DA, Dorsal aorta. PCV. Posterior Cardinal Vein. SeA. Segmental artery. SeV. Segmental vein. White arrowheads denote SeA. White arrows denote SeV. Embryos were submitted to microangiography. F. Wild type *vegfc^{um18}* embryo. G. Homozygous *vegfc^{um18}* mutant embryo. **H.** Quantification of percent artery and vein connections in phenotypically wild type and *vegfc^{um18}* mutant embryos. Embryos were scored for the formation of PHBC at 30 hpf. Those displaying a PHBC were denoted wild type, those with absence of PHBC were denoted *um18* mutants. At 3dpf. embryos of both phenotypic classes where scored for the connection of intersegmental vessel to either DA or PCV was based blood flow, starting from the anterior fifth somite onward. Intersegmental vessels returning blood flow into the DA were denoted as an arterial connection. Intersegmental vessels returning blood flow into the PCV were denoted as a venous connection. The values shown are based on the average of three independent experiments.

intersomitic vein formation and found that in comparison to wild type siblings, homozygous *vegfc*^{um18} mutants had significantly less secondary intersomitic vein connections (Figure 7F-H), although *vegfc*^{um18} heterozygotes appeared normal in this regard (personal observation). These data suggest that the primary defect in *vegfc*^{um18} mutants is a failure in venous and lymphatic progenitor sprouting.

Our data suggest an early defect in sprouting is the primary defect in *vegfc*^{um18} mutants. To determine whether Vegfc/Flt4 signaling was required for subsequent events in lymphatic pattering, we blocked Flt4 activation at separate time points before and after PL sprouting using a kinase inhibitor of Flt4 (MAZ-51). To better visualize the TD in treated embryos we used the Tg(*flk1*:crgfp)^{zn1};Tg(*fliep*:dsRedEX)^{um13} double transgenic line, which labels the TD red. Tg(*flk1*:crgfp)^{zn1};Tg(*fliep*:dsRedEX)^{um13} embryos were separately treated with and subsequently maintained in MAZ-51 at 1,2, 3, and 4 dpf. Treated embryos were assessed for TD formation at 5 dpf. We found that inhibition of Flt4 signaling at 1, 2, 3 and 4 dpf significantly impaired the ability of embryos to form a complete TD when compared to vehicle treated siblings (Figure 8A-B, TD labeled Red). Taken together, these data demonstrate a requirement for the C-terminal domain of Vegfc during sprouting of lymphatic progenitors and segmental vein morphogenesis. Furthermore, these data suggest the reiterative use of Vegfc/Flt4 signaling throughout lymphatic vessel patterning.

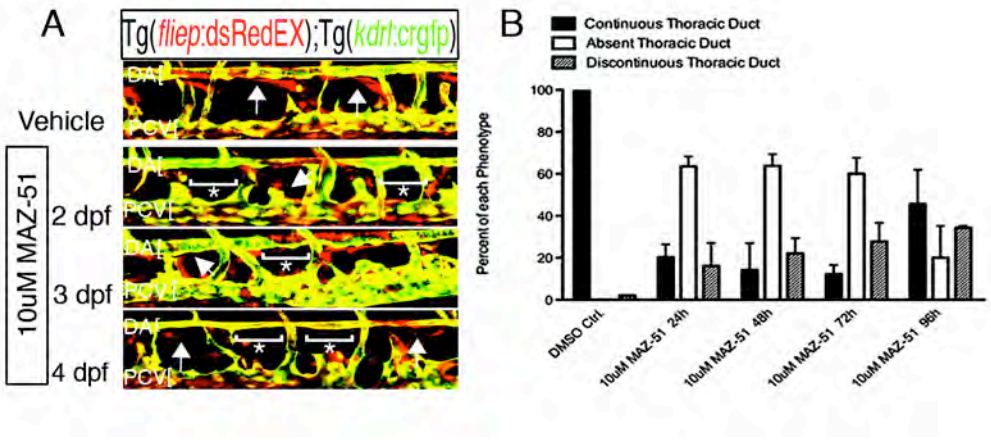


Figure 8.II Vegfc/Flt4 signaling is used reiteratively during lymphatic vessel formation.

(A). Confocal micrographs of trunk blood vessels in Tg(*flk1*:crgfp)^{zn1} ;Tg(*fliep*:dsRedEX)^{um13} at 5 dpf. Red vessels are denoted as a TD by expression of the *fliep*:dsRedEX transgene. Yellow vessels are denoted blood vessels by colocalization of the *flk1*:crgfp and *fliep*:dsRedEX transgenes. Anterior is to the left dorsal is up. DA, Dorsal aorta. PCV. Posterior Cardinal Vein. Thoracic Duct (TD). Embryos were treated in 10uM MAZ-51 at 24 hpf, 48 hpf, 72 hpf. and 96 hpf. Control embryos were treated in 0.01% DMSO and subsequently assayed for the formation of the TD at 120 hpf. **(B)** Quantification of TD in embryos displaying a Continuous TD, Partial TD or Absent TD at 120 hpf. Embryos for each treatment were scored for the above-mentioned phenotypes and compared to the total amount of embryos scored per treatment to calculate the percent of embryos displaying the observed phenotype. Values shown are based on the average of three independent experiments.

Discussion

Vegfc/Flt4 signaling is required for various aspects of vascular development in zebrafish. We hypothesized that screening for early defects in vein morphogenesis in mutant haploid embryos would lead to the identification of genes that are required for Vegfc/Flt4 signaling. Indeed, our findings demonstrate the ability to efficiently carry out a transgenic haploid screen in zebrafish to identify mutants with vein and lymphatic defects. Here, we identified and positionally cloned a truncation allele of *vegfc* that displays vein and lymphatic vessel patterning defects but overtly normal segmental artery formation.

Previously, work by others has relied of the use of F3 ENU mutagenesis screens performed in the Tg(*fli1a*:egfp)^{y1} background to isolate mutants that effect lymphatic vessel patterning [15,34]. Although this standard F3 crossing scheme has yielded mutants with lymphatic defects, the amount of time, space, and labor involved is prohibitive for a small lab group. Here we show that screening for early defects in vein development in mutant transgenic haploid embryos expressing Egfp in endothelial cells can be used as a way to isolate mutants with defects in Vegfc/Flt4 signaling. Importantly, this method of screening reduced the number of generations in our screen by one, thus reducing the amount of time it takes to recover embryos with a given recessive trait. Similar to previous haploid screens[45] we observed that the putative

haploid phenotype was recapitulated in diploid embryos, which further demonstrates that this method of screening can be used to identify mutants affecting vascular development.

We previously used this same haploid strategy in a blood vessel transgenic background to isolate mutants with defects in SeA formation. This screen yielded 17 mutant loci of approximately 1200 mutagenized genomes. The authors were able to establish 14 F2 families of the 17 mutant loci of which only 10 recapitulated the haploid mutant phenotype. These 17 mutants encompassed four distinct classes of segmental artery phenotypes [45]. By contrast, our screen focused on the specific absence of the PHBC and we observed 4 mutant loci of approximately 400 mutagenized genomes; of which we were only able to establish 1 F2 family. This F2 family yielded F3 progeny that recapitulated the observed mutant haploid phenotype. As a result, one of the main differences we observed between this and the aforementioned haploid screen was in the recovery of F2 families (82%, previous vs. 25%, present). Given that both screens focused on particular aspects of vascular development; one on SeA formation [14] and the other PHBC formation (present study) we can speculate that if we were able to recover a higher proportion of F2 families then we would increase the rate of identifying PHBC mutants.

Although we were able to successfully identify a lymphatic mutant using this indirect screening approach, this method of screening would possibly fail to obtain genes that are involved in lymphatic vessel development but not PHBC

formation. Some of these examples include: the calcium and collagen binding EGF-like domain 1 gene (*cceb1), synectin, sox18* and *prox1*, all of which display lymphatic defects but normal PHBC formation (personal observation)[34,53]. Additionally, this method of screening could potentially fail to identify genes involved in aspects of vascular development that are required past the formation of the PHBC, therefore excluding genes involved in lymphatic vessel function and maintenance for example. However, during the characterization of the *vegfc*^{um18} mutant we noted that the Flt4 signaling pathway was used reiteratively throughout the formation of the TD (Figure 8A-B). This implies that indirectly screening for early defects in vein morphogenesis, which requires Vegfc/Flt4 signaling, may be useful to identify additional mutant alleles that affect aspects of lymphatic vessel development in zebrafish.

The profound defects of the *vegfc*^{um18} mutation on lymphatic vessel development suggest that the C-terminal domain of Vegfc is necessary for proper vein and lymphatic vessel formation but dispensable for segmental artery formation. Our finding that that *vegfc*^{um18} mutants display general defects in venous and lymphatic progenitor cell sprouting were strikingly similar to those observed in *Vegfc* knockout mice and Vegfc morphant zebrafish embryos, where lymphatic progenitor cells fail to sprout from the PCV [16,17,33]. Why are lymphatic defects so severe in *vegfc*^{um18} mutants? Further insight may come from biochemical and molecular characterization of Vegfc^{um18}, which

include investigation into its secretion and ability to activate Flt4. Structurally the C-terminal domain of Vegfc is similar to the secretory silk protein, BR3P. In addition, it also contains short motifs homologous to EGF-like domains, which have been known to associate with the extracellular matrix (ECM) [59,60]. However, to this date no function has been ascribed to this domain.

We observed a haploinsufficient lymphatic vessel phenotype in *vegfc*^{um18} mutants. This phenotype was reminiscent of *Vegfc* heterozygous knockout mice, which display developmental and functional lymphatic vessel defects. Therefore, our data suggests that *vegfc*^{um18} may act as a null allele during lymphatic vessel development. Interestingly, we were able to recover visibly healthy heterozygous *vegfc*^{um18} carriers at the expected frequency suggesting recovery of the lymphatic defects. Therefore, it is possible that the TD is delayed in *vegfc*^{um18} heterozygotes and recovers later in development. Yet another possibility may be the existence of an additional factor, which may act redundantly to pattern the TD. Vegfd is member of the Vegf family that has been shown to activate Flt4. In mice, recombinant Vegfd was able to rescue migration in *vegfc* deficient lymphatic cells [16]. However, similar to mice, endogenous *vegfd* is not expressed near areas of TD formation in zebrafish (personal observation).

In zebrafish, *vegfc* is normally expressed in the dorsal aorta. Recently a study in this model system has described the requirement of arterial endothelial cells in the formation of lymphatics [36]. Consistent with these studies, expression of

vegfc in arterial cells rescued lymphatic vessel formation in *vegfc*^{um18} mutants (Figure 5D-F and J). This expression was adjacent to where the TD ultimately forms. Additionally, similar expression of *vegfc*^{um18} failed to rescue lymphatics in affected embryos homozygous mutant embryos. However, we noted a partial rescue of lymphatics in *vegfc*^{um18} heterozygotes, implying that increased levels of *vegfc*^{um18} in the presence of a wild type copy of *vegfc* is enough to pattern lymphatics. Taken together, these data imply that *vegfc*^{um18} mutants may have paracrine defects, which ultimately perturb budding of lymphatic progenitors. These data also support a paracrine mode of signaling by Vegfc from blood vessels in the formation of lymphatics during development. However, further characterization of Vegfc^{um18} should help uncover the molecular basis for defects in *vegfc*^{um18} mutants.

Finally, we have previously demonstrated that *vegfc* is required for proper segmental artery morphogenesis in zebrafish. Interestingly, segmental arteries develop normally in *vegfc*^{um18} mutants. Why is segmental artery formation normal in *vegfc*^{um18} mutants? Since *vegfc* is expressed in SegA, it is possible that *vegfc*^{um18} may still be active in this context of vascular development, independent of the Vegfc C-terminal domain. Further biochemical and genetic analysis of *vegfc*^{um18} presented in the proceeding chapter will shed light into the role of Vegfc during developmental angiogenesis.

In summary, we successfully employ a transgenic haploid screen to identify mutants that affect distinct aspects of vascular development through Vegfc/Flt4

signaling. The mutant allele identified in this study helped revealed a previously unknown requirement for the C-terminal domain of Vegfc during vein and lymphatic vessel sprouting. The non-cell autonomous rescue of lymphatics by wild type *vegfc* demonstrates a paracrine signaling mechanism of Vegfc by the dorsal aorta to pattern the lymphatics during embryonic development. Interestingly, the C-terminal domain appears to be dispensable during developmental angiogenesis. Together, these data highlight the diverse role of Vegfc/Flt4 signaling during vascular development and imply a context dependent role for this signaling pathway during formation of distinct vessel types in zebrafish.

CHAPTER III

GENETIC, BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF *VEGFC*^{UM18} REVEALS DISTINCT MODES OF SIGNALING DURING BLOOD AND LYMPHATIC VESSEL DEVELOPMENT IN ZEBRAFISH.

Introduction

In the preceding chapter we identified and cloned a mutant allele of *vegfc* (*vegfc*^{um18}), which results in the premature truncation of zebrafish Vegfc and eliminates its C-terminal domain. Affected embryos lack lymphatic vessel formation due to earlier defects in parachoradal lymphangiobalst (PLs) and venous sprouting. We found that transient transgenic expression of *vegfc* in the DA rescued lymphatic vessel formation as indicated by presence of the thoracic duct (TD) in mutant embryos. Interestingly, we observed that segmental arteries (SegA) developed normally in *vegfc*^{um18} mutants when compared to their wild type siblings. Our results suggest the C-terminal domain of Vegfc is required for certain aspects of vascular development (i.e. vein and lymphatic vessels) yet dispensable for others (i.e. SeA formation), thus emphasizing the diverse role of the Vegfc/Flt4 signaling pathway during vascular development.

Vegfc is a secreted growth factor that undergoes proteolytic processing events that regulate its activity. As mentioned in the preceding chapter, sites of processing reveal domains within Vegfc including a central Vegf homology domain (VHD), which is separated from the C-terminal domain by a conserved Furin motif. The VHD has been shown to be necessary and sufficient for receptor activation, while the function of the C-terminal domain is largely unknown[60,74]. The *um18* mutation creates a truncated version of Vegfc with an intact VHD but absent C-terminal domain. Structurally, the C-terminal domain of Vegfc is similar to the secretory silk protein, BR3P, and contains short motifs that are

homologous to EGF-like domains of other secreted proteins [58,59,66,114]. These short motifs are involved in protein secretion and interactions with the extracellular matrix (ECM) [73,114].

Genetic evaluation of *vegfc*^{um18} mutants suggest that the observed phenotype during vein and lymphatic vessel formation may be due to a functional failure of Vegfc/Flt4 signaling. This phenotype is consistent with previous *vegfc*/*flt4* loss of function studies in vertebrates, which have demonstrated that proper functioning of this signaling pathway is necessary for vein and lymphatic vessel formation[8,15,16,33]. Interestingly, *vegfc*^{um18} mutants do not phenocopy *vegfc/flt4* loss of function during developmental angiogenesis[14,62] implying that Vegfc/Flt4 signaling may still be functional in *vegfc*^{um18} mutants in this context. Further insight into to the mechanisms that regulate the formation of these distinct vessel types during development may come from evaluating how the *vegfc*^{um18} mutation affects the molecular function of Vegfc.

In this chapter we present evidence that suggests two modes of signaling by Vegfc during blood and lymphatic vessel development in zebrafish. We find that inefficient secretion is the primary molecular defect of Vegfc^{um18}, thus implying that the observed lymphatic defects in *vegfc*^{um18} is due to inadequate paracrine activity by Vegfc. By contrast, during SeA formation, we observe functional redundancy from the Vegfa signaling pathway that likely compensates for the secretion defects in *vegfc*^{um18} mutants. Additionally, we find that Vegfc appears to act in an autocrine manner during blood vessel development. We observed that

Vegfc^{um18} induced mild effects on blood vessels when expressed in an endothelial autonomous manner. Moreover, we find that cell autonomous downregulation of *vegfc* in endothelial cells reduces their ability to occupy the tip cell position during developmental angiogenesis, suggesting a cell autonomous/ autocrine role for Vegfc during developmental angiogenesis. As a whole, we believe our results suggest a possible mechanism that involves discrete modes of cell signaling by the Vegfc/Flt4 pathway in the formation of blood and lymphatic vasculature in zebrafish.

Materials and Methods

Zebrafish, transgenic and mutant lines

Zebrafish were housed and maintained in accordance to standard protocols described elsewhere. The *um18* is stop codon mutation in the fourth exon of *vegfc* and was isolated from a haploid mutagenesis screen. *um18* mutant carriers were crossed in the Tg(*fli1a*:egfp)^{y1} background. Wild type Tg(*fli1a*:egfp)^{y1} zebrafish embryos were used for ZFYM assay and mammalian cell xenograft studies.

Phenotypic analysis and microscopy of zebrafish embryos.

Imaging general morphology of zebrafish using transmitted light was performed on a Leica MZ FLIII using a Zeiss AxioCam MRc digital camera. Images of blood vessels, subintestinal vessels (SIVs) and xenografted NIH3T3 cells expressing either ligand in Tg(fli1a:egfp)^{y1} embryos were obtained using a Leica DMIRE2 confocal microscope (Objective: HC PL APO 20x/ 0.70CS). To measure segmental artery length, embryos were fixed and subjected to antibody staining using an α-EGFP antibody (Molecular Probes) [14]. Images of segmental arteries were captured using confocal microscopy and length was quantified using Imaris software (Bitplane).

Conditioned zfVegfc and zfVegfc^{um18} media.

The pMT-Ex-zfVEGF-C-wt vector for the expression of full-length wild type zebrafish VEGF-C in Drosophila S2 cells (Invitrogen) was constructed by cloning the BamHI/NotI-cleaved PCR product forward primer: 5'

GAGGATCCATTCGAGTCAAGTCACGACTAC-3', reverse primer: 5'-GGGCCCTCTAGACTCGAGCG-3', template: full-length zebrafish VEGF-C cDNA (Ref1) into a pMTBiP-V5His-C vector (Invitrogen), that had been modified to a) include the hygromycin resistance gene from pCoHygro (Invitrogen) and b) have a frameshift mutation between the coding sequence for the BiP signal peptide and the multiple cloning site such that the 5'-GAT-3' from the BglII site codes for an aspartic acid residue. The construction of pMT-Ex-zfVEGF-C-um18 was done in the same way except that the reverse primer was 5'-CTGCGGCCGCTTATTTTGACAAACAGCTGCAGGAAG-3'. S2 cells were transfected with the constructs and stable cell pools were created according to standard protocols. To produce protein, cells were induced with 1 mM CuSO4 and conditioned cell supernatant was harvested 3.5 days post induction. **These media were produced by Michael Jelstch in a collaboration with the Alitalo Lab (Univ. of Helsinki)**

Zebrafish yolk membrane angiogenesis assay (ZFYM) and Mammalian cell/zebrafish xenografts.

We separately injected 4nL of normalized zebrafish Vegfc (zfVegfc), zfVegfc^{um18} and negative control conditioned media into the perivitellene space of anesthetized 48 hpf Tg(Fli1a:egfp)^{y1} embryos as described elsewhere[115]. Injected embryos were assayed for the total amount of ectopic vessels sprouting from SIVs at 3.5 dpf. Images of embryos were obtained using a Leica DMIRE2

confocal microscope (Objective: HC PL APO 20x/ 0.70CS) and analyzed using the Imaris imaging software package (Bitplane).

For the xenograft studies, we anesthetized 48 hpf embryos injected with ~1500 cells/nL stable NIH 3T3 cells expressing either *zfvegfc-2Acherry* or *zfvegfc*^{um18}*-2Acherry* diluted in matrigel (Sigma). Xenografted embryos were allowed to develop 2 days post engraftment and subsequently evaluated for neovacularization of grafted cells. Imaris imaging software (Bitplane) was used to quantify the relative amount of ligand expressing cells (red voxels) and vessels (green voxels)[116].

VEGF-C secretion analysis in cultured cells

NIH3T3 cells cultured in DMEM supplemented with 10% newborn fetal calf serum were transiently transfected with either pSport6CMVVEGF-C (Open Biosystems) or pCSVEGF-CΔC. To generate the VEGF-CΔC expression vector we generated a VEGF-CΔC Gateway entry clone as described elsewhere. We used the pSport6CMVVEGF-C plasmid as a template and amplified VEGF-CΔC using the following primers: 5' GGGGACAAGTTTGTACAAAAAAGCAGGCTGGGCCACC**ATG**CACTTGCTGG GCTTCTTCT 3' and 5' GGGGACCACTTTGTACAAGAAAGCTGGGTCTTATTTAGACATGCATCGGCA GG 3'. pME VEGF-CΔC was LR cloned into the pCSDest plasmid to create the pCSVEGF-CΔC. Cells were collected at 30 hour post transfection and 72 hours post transfection and subsequently lysed in RIPA lysis buffer (RIPA- 150mM

NaCl, 50mM Tris ph 8.0, 1% NP-40, 0.1%SDS, Complete Mini (Roche)). Media was collected and concentrated using centrifugal filter units (Ultragel -10K, Millipore). Lysates and media were subjected to SDS-PAGE immunoblot analysis and probed with a polyclonal human anti-VEGF-C antibody (provided by the Alitalo). Membranes were stripped and re-probed with a monoclonal α-Tubulin (Green Lab) antibody as a loading control.

Flt4 activation assay

NIH3T3 cultured in DMEM supplemented with 10% newborn fetal calf serum were transiently transfected with pCSzfflt4-HA. At 24 hrs post transfection, cells were starved for 24 hrs and separately stimulated with normalized zfVegfc, zfVegfc^{um18}, and negative control conditioned media. Cells were lysed in RIPA lysis buffer and submitted to immunoprecipitation analysis using a polyclonal antibody raised to the zebrafish homologue of VEGFR3 (zfFlt4). Immuoprecipitated samples were subsequently submitted to SDS-PAGE immunoblot analysis and probed with a monoclonal antibody raised to phosphoTyrosine (4G10 clone, Millipore). Membranes were stripped and re-probed with a monoclonal α-HA (BABCO) to normalize against total zfFlt4. To generate pCSzfflt4-HA, we created pMEflt4 w/o stp using the forward 5'-GCCACCATGAAGAGAGATTTTACGTTTTTCTG-3' and reverse: 5'-CGTAAACGGCCTGGTCTGAG-3'. pMEflt4 w/o stp and p3E-HA were cloned into pCSDest2 using LR clonase plus II.

Genotyping

To genotype Tg(*fli1a*:egfp)^{y1}*vegfc*^{um18};*kdr*^{y17} double mutants, DNA was extracted as described elsewhere. The *kdr*^{y17} allele was genotyped using the dCAP oligos (ref.) followed by DdeI restriction enzyme digest. To genotype the *vegfc*^{um18} allele, DNA was extracted as described elsewhere and subjected to a customized TaqMan SNP genotyping assay (Applied Biosystems).

Zebrafish DNA injections

To analyze the autonomous effect of *vegfc* and *vegfc*^{um18} on blood vessels we injected one-cell stage Tg(*fli1a*:egfp)^{y1} embryos with 25pg of either pTol2*flieb:vegfc-2Amcherry*, pTol2*flieb:vegfc*^{um18}*-2Amcherry* or *pTol2fliebsegfp-2Acherry* together with 25pg of *tol2 transposase* mRNA. We subsequently counted the amount of ectopic vessel of mCherry expressing segmental arteries over the span of six hemisegments.

Mosaic Analysis

For mosaic analysis of *vegfc* deficient cells in wild type vasculature, we injected either *vegfc* MO or control MO into Tg(*fli1a*:egfp)^{y1} embryos at 1-2 cell stage and allowed to develop. At the high stage, cells from the marginal zone were separately extracted from *vegfc* deficient and control MO injected Tg(*fli1a*:egfp)^{y1} embryos and transplanted into Tg(*kdrl:rasmcherry*)^{s916} host embryos at the same stage. Embryos were allowed to develop until 30 hpf. We subsequently noted the position of the transplanted vegfc MO or control MO endothelial cells (green) in the wild type Tg(*kdrl:rasmcherry*)^{s916} host. We noted contribution to the tip cell,

stalk, dorsal aorta or posterior cardinal vein in both control and *vegfc* MO transplanted cells.

Results

The C-Terminal domain of Vegfc is required for its secretion

To better understand how the *um18* mutation affects the molecular function of Vegfc, we first tested the ability of wild type and mutant Vegfc to activate Flt4. In mammalian cells expressing the zebrafish homolog of Flt4, we observed that Vegfc-conditioned media potently induced autophosphorylation of Flt4 compared to both the negative conditioned media and PBS treated controls (Figure 1A lanes 1,2 and 4). Likewise, we observed that Vegfc^{um18}-conditioned media induced Flt4 activation to levels similar to wild type Vegfc (Figure 1A, compare lane 2 and 3). As a parallel *in vivo* assay, we compared the activity of wild type Vegfc and Vegfc^{um18} using the zebrafish yolk angiogenesis assay (ZFYM)[115]. Following injection of Vegfc-conditioned media into the perivitelline space of zebrafish embryos at 48 hpf, we observed significant induction of ectopic sub intestinal vessels (SIVs) when compared to uninjected embryos or those injected with mock conditioned media (Figure 1B-E). Additionally, we found that Vegfc^{um18}-conditioned media induced ectopic SIV formation similar to levels induced by Vegfc (Figure 1D-F). Together, these observations indicate that loss of the C-terminal domain of Vegfc caused by the *um18* mutation does not affect its ability to activate Flt4 or to directly induce an angiogenic response *in vivo*.

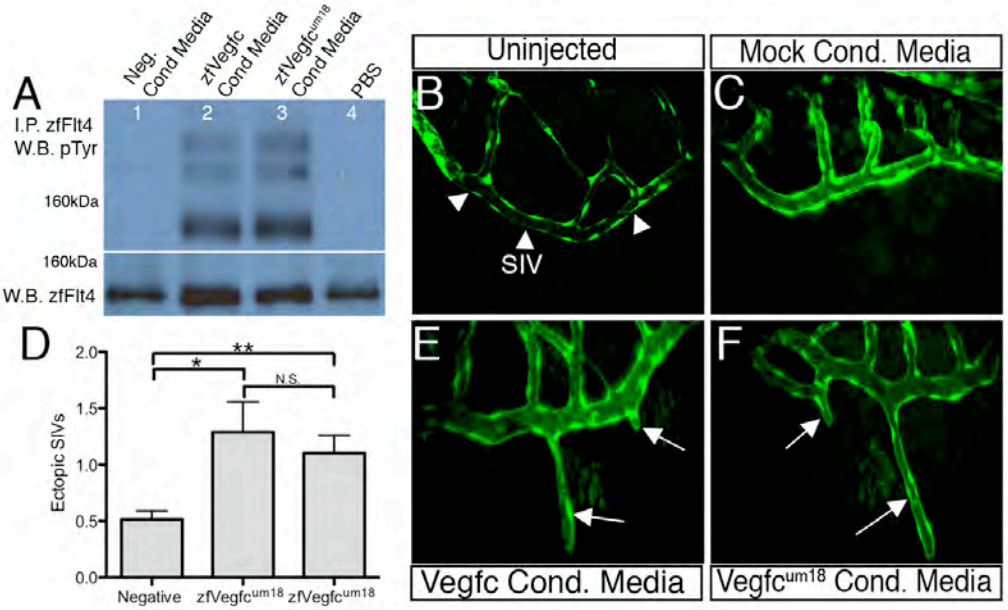

A
Neg. Cond Media
zfVegfc Cond Media
zfVegfc^um18 Cond Media
PBS
1
2
3
4
I.P. zfFlt4
W.B. pTyr
160kDa
160kDa
W.B. zfFlt4
D
Ectopic SIVs
2.0
1.5
1.0
0.5
0.0
**
*
N.S.
Negative
zfVegfc^um18
zfVegfc^um18
Uninjected
Mock Cond. Media
B
C
SIV
E
F
Vegfc Cond. Media
Vegfc^um18 Cond. Media

Figure 1.III Vegfc^{um18} induces zfFlt4 activation and *in vivo* angiogensis.

(A) Phosphorylation of zebrafish Flt4 (zfFlt4) by wild type zebrafish Vegfc (zfVegfc) and zebrafish Vegfc^{um18} (zfVegfc^{um18}). Mouse fibroblasts (NIH 3T3) transiently expressing zfFlt4 and starved for 24 hrs were treated separately with conditioned Negative control, zfVegfc, and zfVegfc^{um18} conditioned media. PBS treated cells were used as control for autophosphorylation. Subsequent immuneprecipitation of lysates with an antibody directed toward zebrafish Flt4 followed by western blot analysis with an anti- phosphotyrosine (pTyr) antibody. Membranes were stripped and probed with zfFlt4 antibody to assess total levels of zfFlt4 in immunoprecipitated lysates. **(B-C, E-F)** *In vivo* zebrafish yolk membrane angiogenesis assay (ZFYM). Confocal micrographs of SIV, Sub intestinal vessels at 3.5 dpf, 1.5 days post injection. Anterior is to the left, dorsal is up. (B) uninjected *Tg(fli1a:egfp)^{y1}* embryo. White arrowheads indicate the Sub-Intestinal Vessels (SIVs). (D) *Tg(fli1a:egfp)^{y1}* embryo injected with Negative control conditioned media. (E) *Tg(fli1a:egfp)^{y1}* embryo injected with zfVegfc conditioned media. (F) *Tg(fli1a:egfp)^{y1}* embryo injected with zfVegfc^{um18} conditioned media. (E-F) White arrows indicate ectopic SIVs. (**G.)** Quantification of ectopic SIVs. Values are shown based on the average of three independent experiments (*, **p< 0.05) (N.S.- not significant).

These observations are consistent with previous studies on human Vegfc in which the Vegf-homology domain alone is sufficient to activate Flt4 [60].

Vegfc is a growth factor that is thought to act in a paracrine manner to induce lymphangiogenesis[1,2,16]. Therefore, we examined whether the C-terminal domain of Vegfc was required for its secretion. Since an appropriate antibody against zebrafish Vegfc was not available, we analyzed lysates and media of mammalian cells transiently expressing human VEGF-C (VEGF-C) or the human equivalent of Vegfc^{um18} (VEGF-CΔC). As expected, we were able to detect the presence of both the unprocessed and processed forms of VEGF-C in lysates and media fractions of transfected cells (Figure 2A, lanes 1 and 3). By contrast, we noted an increased accumulation of VEGF-CΔC in cell lysates compared to wild type VEGF-C (Figure 2A, lanes 1 and 2) and we were unable to detect any VEGF-CΔC in the media from expressing cells at 30 hours post transfection (Figure 2A, lane 4, and 2B), although by 72 hours post transfection, media from mammalian cells expressing VEGFC-CΔC displayed levels of secretion similar to those observed in media of mammalian cells expressing VEGFC. (Figure 2C, compare lane 3 and 4). Evaluation of lysates of both VEGFC or VEGFC-ΔC expressing cells at this time point revealed that VEGFC-ΔC continued to accumulate in cell lysates (Figure 2C, lane 2). By contrast VEGFC was efficiently secreted, as it was barely detectable in the cytoplasmic fraction but at high levels in the media (Figure 2C lanes 1 and 3), implying that VEGFC-ΔC is inefficiently secreted.

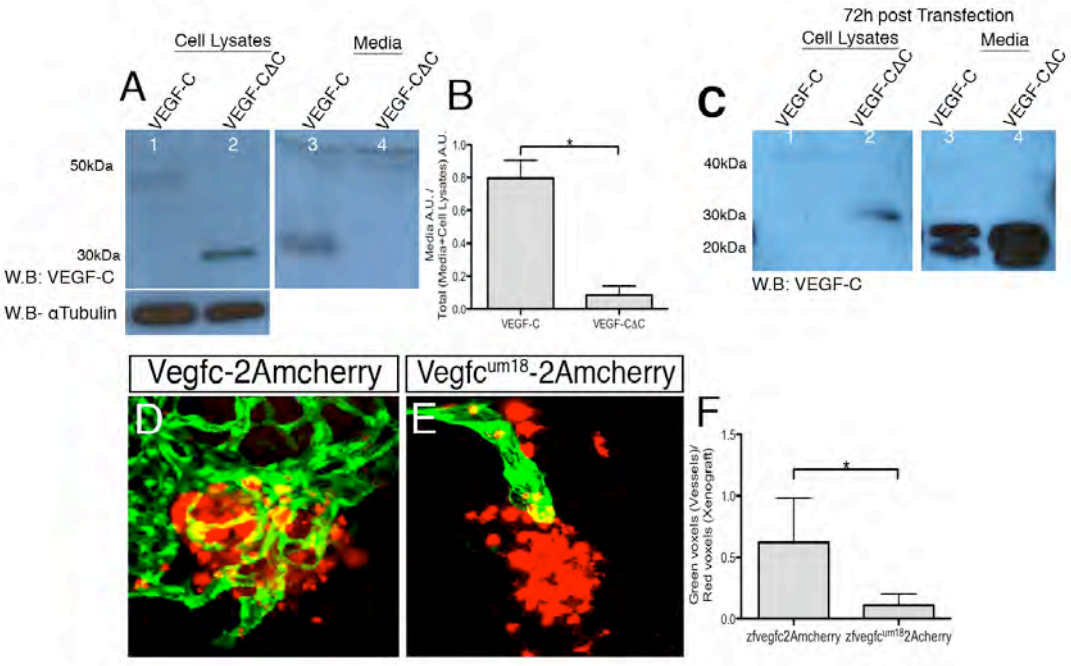

A
Cell Lysates
Media
VEGF-C
VEGF-CΔC
VEGF-C
VEGF-CΔC
1
2
3
4
50kDa
30kDa
W.B: VEGF-C
W.B- αTubulin
B
Media A.U./
Total (Media+Cell Lysates) A.U.
1.0
0.8
0.6
0.4
0.2
0.0
*
VEGF-C
VEGF-CΔC
C
72h post Transfection
Cell Lysates
Media
VEGF-C
VEGF-CΔC
VEGF-C
VEGF-CΔC
1
2
3
4
40kDa
30kDa
20kDa
W.B: VEGF-C
Vegfc-2Amcherry
Vegfc^um18-2Amcherry
D
E
F
Green voxels (Vessels)/
Red voxels (Xenograft)
1.5
1.0
0.5
0.0
*
zfvegfc2Amcherry
zfvegfc^um18^2Acherry

Figure 2.III Vegfc^{um18} lacks the ability to be secreted and induce angiogenic response *in vivo*.

(A-B) Biochemical evidence that the C-terminal domain of Vegfc is required for its secretion. Cell lysates and Media from NIH3T3 cells transiently expressing human vegfc (VEGF-C) and human equivalent of vegfc^{um18} (VEGF-CΔC) separately, were used to monitor both VEGF-C and VEGF-CΔC secretion. Cytoplasmic and Media fractions were collected 30 hrs post transfection and subsequently analyzed by immunoblot with VEGF-C antisera. Western blot analysis of total cell lysate was performed with an anti- α Actin antibody. (H) Quantitative analysis of VEGF-C and VEGF-CΔC secretion. The histogram is a quantification of western blot analysis (A) where the amount of VEGF-C and VEGF-CΔC secretion was expressed as a ratio of Media arbitrary units (A.U.) over the Total (Media and Cell Lysate fractions) A.U. Values are shown based on the average of three independent experiments (*p<0.05). **(C)** Cell lysates and Media from NIH3T3 cells transiently expressing human vegfc (VEGF-C) and human equivalent of vegfc^{um18} (VEGF-CΔC) separately. Cytoplasmic and Media fractions were collected 72h hrs post transfection and subsequently analyzed by immunoblot with VEGF-C antisera. **(D-F)** *in vivo* zebrafish xenograft assay. Confocal image of a 4.5dpf Tg(fli1:egfp)^{y1} where NIH3T3 cells expressing either Vegfc-P2Amcherry or Vegfc^{um18}-P2Amcherry were xenografted in to the perivitellene space of the embryos at 2.5dpf and subsequently investigated invasiveness of the xenograft by blood vessels at 4.5dpf. Anterior is to the left, dorsal is up. (D) Tg(fli1:egfp)^{y1} with xenografted NIH3T3 cells expressing Vegfc-P2Amcherry. (E) Tg(fli1:egfp)^{y1} with xenografted NIH3T3 cells expressing Vegfc^{um18}-P2Amcherry. (F) Quantification of zebraffish xenograft assay. Where invasiveness of the xenograft was measured as a ratio of green voxels (blood vessel) over red voxels (mammalian cells) expressing. Values are shown based on the average of three independent experiments (*p<0.05).

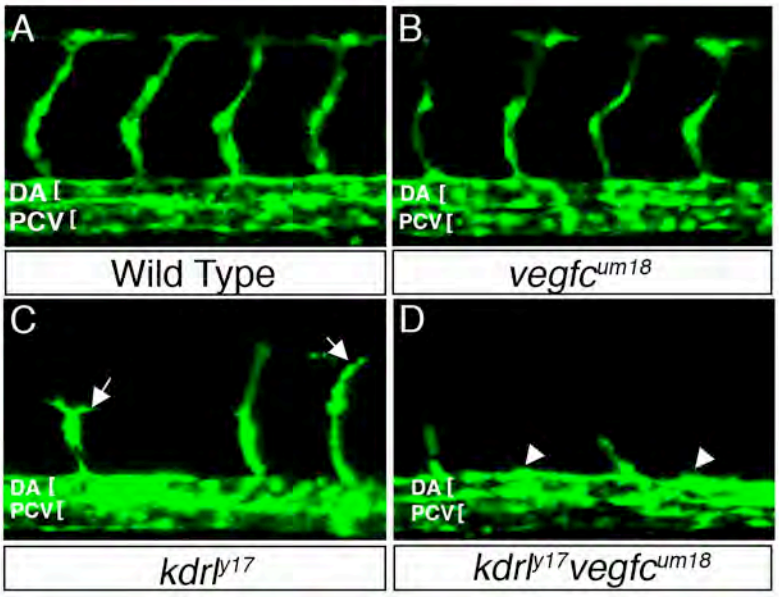
These results suggest that a defect in Vegfc secretion is likely responsible for the lymphatic defects in *vegfc*^{um18} mutants.

To further confirm the consequence of this secretion defect *in vivo*, we assessed the ability of xenografted cells expressing either zebrafish *vegfc* or *vegfc*^{um18} to induce blood vascular growth in zebrafish embryos. As above (Chapter II), both *vegfc* and *vegfc*^{um18} were fused to *2Amcherry* to visualize ligand-expressing cells. At 2 days post graft we noted that mock-transfected cells were unable to induce appreciable levels of vascularization, while those expressing Vegfc-2Amcherry were highly vascularized (data not shown and Figure 2D). By contrast, grafted cells expressing Vegfc^{um18}-2Amcherry induced significantly lower levels of neovascularization (Figure 2E-F). Taken together, these data suggest that the C-terminal domain of Vegfc is required for its efficient secretion and ability to act in a paracrine manner to induce neovascularization and lymphatic vessel formation.

A role for Vegfc signaling during developmental angiogenesis.

We have previously shown that genetic downregulation *vegfc* impairs SeA sprouting in zebrafish embryos, showing [14]. However, we did not observe significant defects in SeA length of *vegfc*^{um18} mutants when compared to wild type siblings (see below). Since *vegfc* is expressed in the endothelial cells of the SeA themselves, it is plausible that Vegfc might act in an autocrine manner, in which case the secretion defect caused by *um18* would have mild or no effects on blood vessel sprouting. Given that SegA formation involves signals from other signaling pathways, it is further plausible that compensatory signals from other

signaling pathways such as Vegfa could mask these effects, i.e. defect associated with *vegfc*^{um18} . Previously, we along with others have shown that the Vegfa signaling pathway is essential for angiogenesis [14,44,57,78]. To determine whether Vegfa signaling compensates for defects in *vegfc*^{um18}, we generated adult zebrafish bearing the *Tg(fli1a:egfp)*^{y1} transgene that were doubly heterozygous for *vegfc*^{um18} and *kdrl*^{y17}, a kinase dead mutation in the zebrafish ortholog of *vegf receptor-2*, *kdrl*, and assessed SeA length in their progeny. In *vegfc*^{um18} homozygous mutant embryos from *Tg(fli1a:egfp)*^{y1} double mutant carriers, we did not observe any significant reduction in SeA length at 30 hpf compared to embryos that were homozygous wild type at both loci (Fig. 3A, B, E). By contrast, we observed variable, but significantly reduced, SeA length in *kdr*^{y17} mutant embryos that were wild type for the *vegfc* locus, similar to our previous work (Fig. 3C, E)[45]. However, in *kdrl*^{y17}*;vegfc*^{um18} double mutant embryos (*vegfc*$^{-/-}$*;kdr*$^{-/-}$), we noted much more severe and significant decreases in SeA length as compared to either wild type or *kdrl*^{y17} mutants alone (Fig. 3A-E). These observations suggest that normal sprouting can proceed in *vegfc*^{um18} mutants as a result of compensatory signaling through the Vegfa pathway. Moreover, these data also imply that Vegfc signaling has marginal effects on blood vessel development, in comparison to its prominent role during lymphatic vessel development.


A
B
DA [
PCV [
Wild Type
vegfc^um18
C
D
kdrl^y17
kdrl^y17vegfc^um18

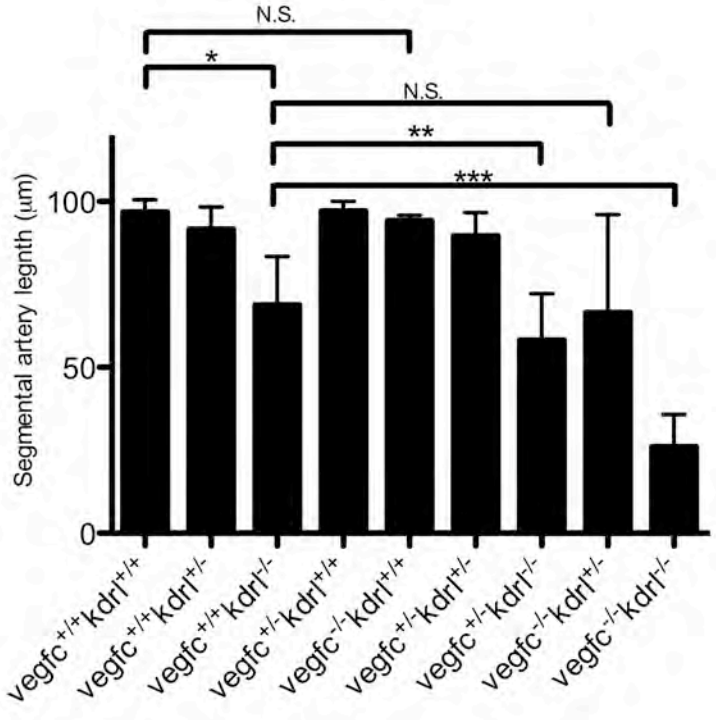

N.S.
*
N.S.
**
***
Segmental artery legnth (μm)
100
50
0
vegfc+/+kdrl+/+
vegfc+/+kdrl+/-
vegfc+/+kdrl-/-
vegfc+/-kdrl+/+
vegfc-/-kdrl+/+
vegfc+/-kdrl+/-
vegfc+/-kdrl-/-
vegfc-/-kdrl+/-
vegfc-/-kdrl-/-

Figure 3.III Vegfa signaling masks defects in *vegfc*^{um18} mutant embryos during developmental angiogenesis.

(A-F) Confocal micrographs of trunk blood vessels at 30 hpf. Anterior is to the left, dorsal is up. DA, Dorsal Aorta. PCV, Posterior Cardinal Vein. (D) Wild type Tg(Fli1:egfp)^{y1} embryo. (D) Tg(Fli1:egfp)^{y1};*vegfc*^{um18} mutant embryo. (E) Tg(Fli1:egfp)^{y1};*kdr*^{y17} mutant embryo. White arrows denote defects in SegA sproouting (F) Tg(Fli1:egfp)^{y1};*kdr*^{y17}*vegfc*^{um18} double mutant embryo. White arrowheads denote severe defects in SeA sprouting. (G) Quantification of SegA length. SegA length was measured across seven SegA for each embryo at 30hpf. Embryos were and subsequently genotyped. Values are shown based on the average of two independent experiments. (*, **,***p>0.05) (N.S.-Not Significant).

An endothelial autonomous role for Vegfc during developmental angiogenesis.

In situ expression analysis revealed that *vegfc* is expressed in the endothelial cells of the SeA themselves [14], similar to *flt4* expression. This endothelial cell autonomous expression of *vegfc* and its receptor suggest an autocrine mode of signaling during developmental angiogenesis. In fact, we find that Vegfc acts in parallel with the Vegfa signaling during angiogenesis (above). To investigate the cell autonomous role for Vegfc during blood vessel development, we first determined the autonomous effect of Vegfc and Vegfc^{um18} on endothelial cells by using the *fli1ebs* element to drive their expression in wild type vasculature. We observed that transient expression of *fli1ebs:vegfc-2Amcherry* in developing segmental arteries could induce ectopic vessel formation within the horizontal myoseptum, as compared to transient expression of *egfp-2Amcherry,* which had no effect (Figure 4A-B and D). Likewise, we observed ectopic vessel formation in segmental vessels expressing *fliebs:vegfc^{um18}-2Amcherry* (Figure 4C). However, *vegfc^{um18}* elicited this effect in a significantly lower proportion of vessels compared to wild type *vegfc* (Figure 4D). Thus, *vegfc^{um18}* is still capable of inducing a modest, albeit reduced, angiogenic effect on endothelial cells in which it is expressed, despite the loss of the C-terminus that is required for proper secretion. This is in stark contrast to its complete inability to induce lymphangiogenesis or rescue the *vegfc^{um18}* phenotype compared to wild type Vegfc (see Chapter III, Figure 6). Our results demonstrate that endothelial-

expressed Vegfc can induce angiogenic cell behaviors, even when its secretion is compromised, suggesting that it acts in an autocrine and/or autonomous manner.

Our results demonstrate that endothelial-expressed Vegfc can induce angiogenic cell behaviors, even when its secretion is compromised, suggesting that it acts in an autocrine and/or autonomous manner. Therefore, to determine if endogenous Vegfc was required in a cell autonomous manner, we performed mosaic analysis. For this purpose, we transplanted cells from donor *Tg(fli1:egfp)*^{y1} embryos injected with Vegfc or control morpholino into *Tg(kdrl:rasmcherry)*^{s916} host embryos. We subsequently assayed the ability of GFP-positive donor cells to occupy either the vein, artery, stalk or tip cell position at 30 hpf. We found that control donor endothelial cells were able to occupy all trunk blood vessel positions (Figure 4E and G) with relatively equal frequency. By contrast, *vegfc*-deficient cells displayed much less frequent contribution to both the distal tip cell position and posterior cardinal vein than control morphant cells (Figure 4F and G). Together with our endothelial over-expression manipulations, these observations suggest that vegfc is required in an endothelial cell autonomous manner for sprouting cell behaviors and efficient angiogenesis.

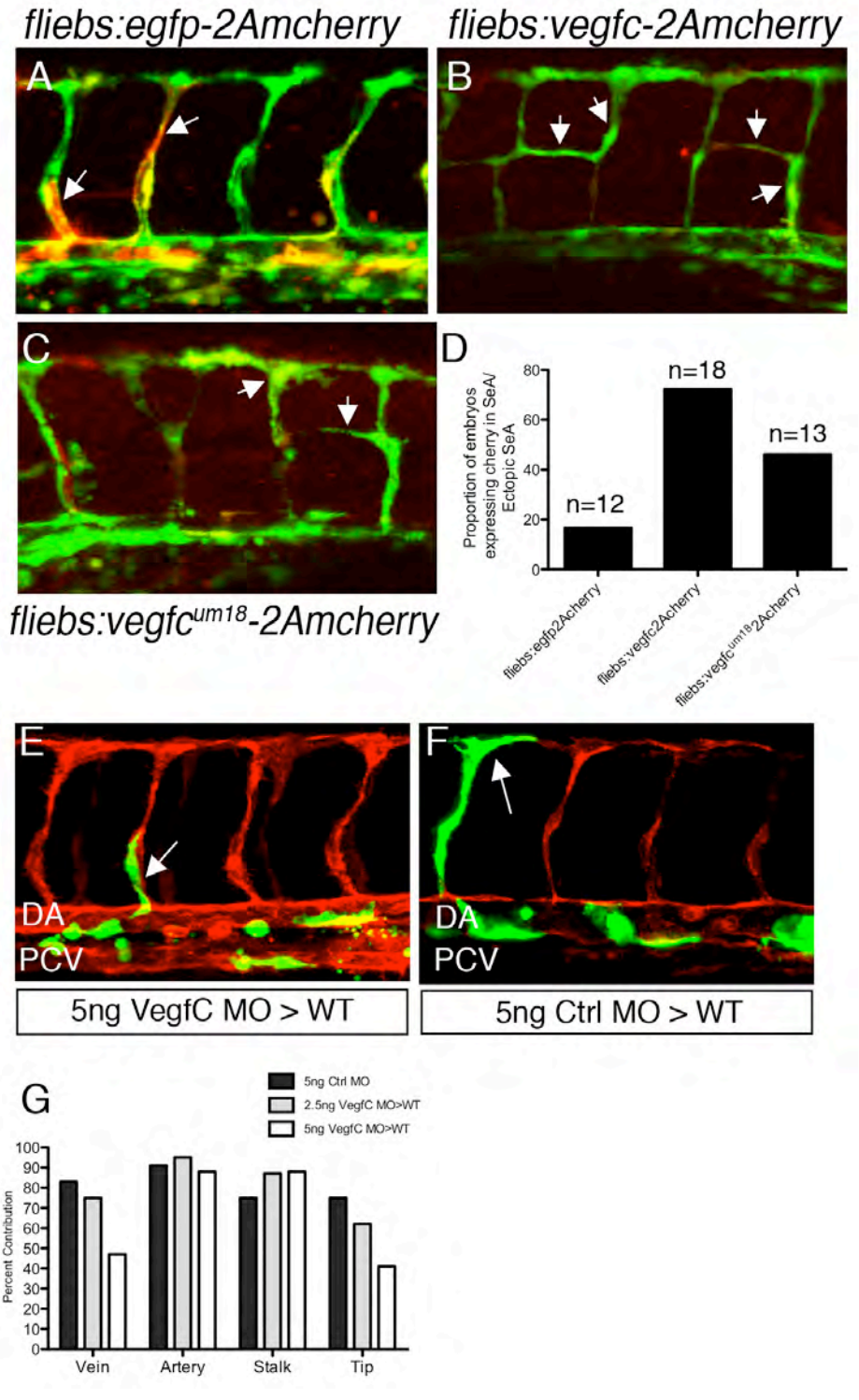

fliebs:egfp-2Amcherry
fliebs:vegfc-2Amcherry
A
B
C
D
fliebs:vegfc^um18-2Amcherry
Proportion of embryos expressing cherry in SeA/ Ectopic SeA
n=12
n=18
n=13
fliebs:egfp2Acherry
fliebs:vegfc2Acherry
fliebs:vegfc^um18-2Acherry
E
F
DA
PCV
5ng VegfC MO > WT
5ng Ctrl MO > WT
G
5ng Ctrl MO
2.5ng VegfC MO>WT
5ng VegfC MO>WT
Percent Contribution
Vein
Artery
Stalk
Tip

Figure 4.III Autocrine role for Vegfc during developmental angiogenesis.

(A-B) Confocal micrographs of trunk blood vessels at 30 hpf. Anterior is to the left, dorsal is up. DA, Dorsal Aorta. PCV, Posterior Cardinal Vein. (A) Tg(*fli1a:*egfp)^{y1} embryos injected with 25 pg transposase mRNA and 25 pg pTol2*fliebs:egfp-2Acherry*. White arrows indicate co-localization of transgene expression in SegA. (B) Tg(*fli1a:*egfp)^{y1} embryos injected with 25 pg transposase mRNA and 25 pg pTol2*fliebs:vegfc-2Acherry*. White arrows indicate co-localization of the transgene in SegA that display ectopic branching. (C) Tg(*fli1a:*egfp)^{y1} embryos injected with 25 pg transposase mRNA and 25 pg pTol2*fliebs:vegfc*^{um18}*-2Acherry*. White arrows indicate co-localization of the transgene in SegA that display ectopic branching. **(D)** Quantification of ectopic branching of SegA in for *fliebs:egfp-2Acherry* (n=12) and *fliebs:vegfc-2Acherry* (n=18) injected embryos expressing the transgene in SeA. Values are shown as proportion of embryos expressing the given transgene within SegA and displaying ectopic branching over the total amount of embryos expressing the given transgene within SeA. NOTE. Parts of this figure appear in Appendix I. **(E-F)** Confocal micrographs of trunk blood vessels at 30 hpf *Tg(kdrl:rasmcherry)*^{s916} host embryos. Anterior is to the left, dorsal is up. DA, Dorsal Aorta. PCV, Posterior Cardinal Vein. (E) *Tg(kdrl:rasmcherry)*^{s916} host embryo transplanted with *vegfc* deficient cells. White arrow denotes a stalk cell. (F) *Tg(kdrl:rasmcherry)*^{s916} host embryo transplanted with Ctrl MO deficient cells. White arrow indicates tip cell position. **(G)** Quantification of endothelial cell occupancy in a wild type host at 30 hpf.

Discussion

In this study we show that inefficient secretion of Vegfc is the primary molecular defect in *vegfc*^{um18} mutants. This lack in paracrine activity is likely responsible for the observed defects in lymphatic vessel formation in this mutant. By contrast, we find that despite Vegfc secretion defects, SeA formation proceeds normally in *vegfc*^{um18} mutants due to compensation by Vegfa signaling. Additionally, we present endothelial cell autonomous loss- and gain-of-function observations, which suggest a cell autonomous autocrine mode of signaling by Vegfc during developmental angiogenesis. As a result these findings reveal distinct endothelial cell autonomous and non-autonomous requirements for Vegfc during blood and lymphatic vessel development in zebrafish.

Our Flt4 activation and *in vivo* angiogenesis studies imply that *vegfc*^{um18} mutants retain the ability to activate Flt4. Together, these observations coincide with those of others showing that the VHD domain of Vegfc is essential and sufficient for activation of both Flt4 and Vegfr-2 [60]. Furthermore, they imply that activation of Flt4 by Vegfc^{um18} is on its own unable to pattern lymphatic vessels and that the C-terminal domain of Vegfc was likely crucial for some aspect of its function that was independent of Flt4 activation. Indeed, further molecular characterization of Vegfc^{um18} revealed that that the C-terminal domain of Vegfc was required for its efficient secretion. This was borne out of our findings that Vegfc^{um18} was inefficiently secreted into the media of mammalian cells expressing its human equivalent (VEGF-CΔC), and that Vegfc^{um18} was unable to induce

neovascularization from xenografted cells expressing Vegfc^{um18}. Therefore, together with our findings that *vegfc^{um18}* mutants display defects in lymphatic vessel formation, which can be rescued by transient transgenic expression of wild type *vegfc* in the DA (Chapter III); we can speculate that the C-terminal silk homology domain of Vegfc plays a significant role in its ability to act as a paracrine factor to guide lymphatics during development. These data also coincide with previous findings in *vegfc* knockout mice, which implied that Vegfc was required in a paracrine manner to induce lymphatic endothelial cell budding of the PCV[16].

Until now the structural domain(s) in Vegfc required for its paracrine activity were unknown. Our findings imply a novel secretory function for the C-terminal domain of Vegfc. Examination of the C-terminal domain of Vegfc reveals conserved tandemly repeated cysteine-rich motifs resembling the silk secretory protein, BR3P[59]. Additionally, this domain contains short EGF-like motifs found in other secreted proteins. These motifs are reminiscent of those contained in latent TGF-β binding proteins (LTBPs). Similar to Vegfc, TGF-β is a proprotein that requires post-translational processing in order to be active. Although, TGF-β has a signal sequence and is translocated to the endoplasmic reticulum after synthesis, it is inefficiently secreted on its own [72,117]. Interestingly, LTBPs have been suggested to augment the secretion of TGF-β [118]. In particular, deletion of the cysteine repeats and or EGF-like domains decreased secretion of TGF-β[119]. Therefore, it is possible that the C-terminal domain of Vegfc acts in a similar fashion to

LTBPs, enhancing Vegfc secretion. In fact, others have found that replacing the heparin binding domain of $Vegf_{165}$, a potent angiogenic isoform of VegfA, with the C-terminal domain of Vegfc increased its angiogenic potential when compared to $Vegf_{165}$ by itself, which has been shown to be the most angiogenic isoform of Vegf [67]. Based on our findings, we can speculate that this is due to an increase in Vegf secretion, conferred by the C-terminal domain of Vegfc. Taken together, our data imply that during lymphatic vessel development Vegfc is secreted by the dorsal aorta and signals in a paracrine mode to induce venous and lymphatic progenitor sprouting as well as guidance of lymphatic vessels. Moreover our data imply that the C-terminal domain of Vegfc enhances Vegfc paracrine activity by increasing its secretion.

Is there a role for Vegfc during developmental angiogenesis? Although the majority of studies involving Vegfc signaling have focused on its role during lymphatic development, several lines of evidence support its function during blood vessel development. First, *vegfc* expression is evident in intersomitic vessels of zebrafish and mice [14,62], suggesting a cell autonomous and/or autocrine mode of signaling during their formation. Second, we have previously shown that genetic knockdown of *vegfc* results in the formation of partial SegA sprouts in zebrafish [14]. However, vascular defects have not been reported in *vegfc* knockout mouse embryos and analyses in zebrafish have been the limited to the aforementioned knockdown technique. Third, studies in zebrafish have suggested that Vegfc and Vegfa signaling genetically interact during blood vessel

formation[14,15]. Lastly, both missexpresion and forced endothelial cell specific expression of Vegfc in mice and zebrafish elicits pro-angiogenic effects [84] (Chapter II). Surprisingly, we did not observe developmental angiogenesis defects in *vegfc*^{um18} mutants, although forced endothelial cell-autonomous expression of Vegfc^{um18} was mildly angiogenic. Our biochemical data suggest that although Vegfc^{um18} is mainly trapped in the cell, it may be secreted at a lower rate. Since *vegfc* is expressed in the endothelial cells of the SeA and cell autonomous downregulation of its expression led to observable angiogenic defects (Figure 4F,G), we hypothesized that Vegfc might act in an autocrine manner, in which case the secretion defect caused by *vegfc*^{um18} would have mild or no effects on blood vessel sprouting. Indeed, our results demonstratethat the Vegfa signaling pathway masked defects caused by Vegfc^{um18}, implying that *vegfc*^{um18} acts as a hypomorph during blood vessel development and is only made severe when Vegfa signaling is disrupted. As result, our findings suggest that Vegfa signaling provides compensatory signals in the absence of efficient Vegfc signaling during developmental angiogenesis. These genetic findings shed light on the interplay between Vegfc and Vegfa signaling during developmental angiogenesis. In particular, Vegfr2 and Flt4 have been shown to form heterodimers and promote angiogenic sprouting upon exogenous Vegfc or Vegfa stimuli, although Vegfc does so more readily[56]. Thus, Vegfc induced heterodimer signaling may be required for some yet unknown aspect(s) of developmental angiogenesis.

In conclusion, our characterization of the *vegfc*^{um18} allele helped uncover two features of Vegfc signaling required for separate processes of vascular development. The C-terminal domain of Vegfc, which is missing in *vegfc*^{um18}, appears to be required for its paracrine activity during lymphatic vessel development. By contrast, our data suggest that during blood vessel development, Vegfc signals in an autocrine manner to drive aspects of developmental angiogenesis. In this context, compensatory signals from Vegfa signaling appear to counteract inefficacies in Vegfc secretion. Interestingly, Vegfc^{um18} is able to induce weak angiogenic effects when expressed in an endothelial cell autonomous manner although endothelial cells deficient for *vegfc* have defects in leading angiogenic sprouting, suggesting a cell autonomous autocrine role for Vegfc during developmental angiogenesis. These findings suggest two modes of signaling by Vegfc/Flt4 signaling in order to regulate discrete aspects of vascular development. In this case a structural domain within Vegfc itself regulates its ability to act in either a paracrine or autocrine fashion leading to its distinct effects on blood and lymphatic vessel development in zebrafish.

CHAPTER IV

GENETIC EVIDENCE FOR THE ROLE OF FURINA IN THE PROCESSING OF VEGFC DURING LYMPHATIC DEVELOPMENT IN ZEBRAFISH

Introduction

Vascular endothelial growth factor c (Vegfc) is a member of the Vegf family of growth factors. Unlike other members of the Vegf family, *vegfc* is translated into a preproprotein, which undergoes complex proteolytic maturation (reviewed in Chapter 1) that ultimately contributes to its ability bind Vegfr3 and Vegfr2[59,60]. Vegfc is made up of an N-terminal signal sequence (SS) followed by N-terminal propeptide domain, a Vegf homology domain (VHD) and a C-terminal cysteine-rich silk homology domain[60](ref.), which we showed in the previous chapter is required for secretion. Examination of the Vegfc amino acid sequence reveals a series of proteolytic cleavage sites that separate its various domains[59,74]. Following cleavage of the SS, the Vegfc propeptide is secreted as a homodimer that contains the C-terminal silk homology domain, which is eventually cleaved in order for it to activate Vegfrs. Others have shown the existence of a conserved dibasic motif (-HSIIRR↓-) separating the VHD from the C-terminal domain of Vegfc. These cleavage motifs are recognized and cleaved by Propetide convertases (PCs), a family of endoproteases [74]. Cleavage at this site in Vegfc has been shown to be required for aspects of vascular development both humans and fin regeneration in zebrafish[74,75].

Furin is a transmembrane PC that cycles between the cell surface and the *trans* golgi network (TGN), its function being the processing of proproteins. Protein precursors such as growth factors, receptors and glycoproteins have all been shown to be substrates for Furin [120-122](ref.). Furin knockout mice are embryonic

lethal, likely due to a failure in processing of factors important for embryogenesis, thus precluding the ability to study its function during embryonic development[76]. The zebrafish genome encodes two duplicates of the *furin* gene (*furinA* and *furinB*). These duplicate *furin* genes have been shown to play a major role during zebrafish craniofacial development [77]. Interestingly, a null mutation in *furinA* displayed less severe skeletal defects than the deficiency in both *furin* genes; thus allowing a more refined analysis of their function during aspects of development.

In the preceding chapters we described the isolation and characterization of a truncation allele of *vegfc*, *vegfc*^{um18}, which completely lacks the Vegfc C-terminal domain. Our studies suggest that this structural domain regulates the ability of Vegfc to signal in either a paracrine or autocrine manner, thus outlining contextual signaling mechanisms by Vegfc during lymphatic and blood vessel development, respectively (Chapters II and III). Inspection of the Vegfc amino acid sequence revealed that the truncation in Vegfc^{um18} occurred prior to the conserved Furin dibasic cleavage motif (-HSIIRR↓-), which separates the VHD from the C-terminal silk homology domain. Cleavage at this site has been shown to be required for aspects of blood vessel development in cultured and tumor cells[74]. However, the *in vivo* biological role of processing at the C-terminal domain of Vegfc during lymphatic patterning remains undetermined. Therefore, investigating whether processing at this Furin cleavage motif is required for lymphatic development may give further insight into the signaling mechanisms

used by Vegfc during lymphatic development. Additionally, structure-function studies have demonstrated that partially processed forms of Vegfc are able to activate Vegfr3 to varying degrees; while in its mature form, Vegfc is able to activate both Vegfr3 and Vegfr2[60]. For this reason, examining the PC(s) involved in processing the C-terminal domain of Vegfc may uncover processing-dependent and/or independent roles for Vegfc during vascular development.

In this chapter, we find that processing of Vegfc at the C-terminal domain is required in order to pattern lymphatics in zebrafish. Furthermore, we present genetic evidence that demonstrate a requirement of *furinA* during lymphatic vessel development but not blood vessel development. Together our findings imply that FurinA cleaves Vegfc in order to activate its function during lymphatic vessel pattering in zebrafish.

Materials and Methods

Zebrafish, transgenic and mutant lines

Zebrafish were housed and maintained in accordance to standard protocols described elsewhere. Wild type EK (Ekkwill Farm derived) and Golden (*gol*^{b1}) embryos were used for whole mount *in situ* hybridization analysis. Tg(*fl1ia*:egfp)^{y1} embryos where used for injections and phenotypic analysis [19](ref). Tg(*fli1ep*:dsredEx);Tg(*nfkb1*:egfp) embryos where used to identify PLs in relation to structures of the lateral line and neuromasts. Tg(*fli1a*:egfp)^{y1};*vegfc*^{um18} mutant line was used to carry out rescue experiments. The TgTP1:d2egfp^{um42};Tg:Fli1ep:dsRedEx^{um13} transgenic line was used to analyze Notch activity in *furinA* deficient embryos.

Construction of non-cleavable *vegfc* (*vegfc*^{SS})

To generate a non-cleavable form of *vegfc* (*vegfc*^{SS}) we specifically mutated the conserved furin cleavage consensus from $_{209}$HIIRR$_{213}$ to $_{209}$HII<u>SS</u>$_{213}$ using overlapping PCR and pME*vegfc* w/o STP as a template(ref.). Mutating these amino acid residues have been shown to abrogate C-terminal cleavage of Vegfc[74,75](ref.). The resulting PCR product was flanked by attB1 and attB2 sites and subsequently BP cloned to pDONR211 to generate pME*vegfc*^{SS} w/o STP. This plasmid was used to generate pTol2*fliebs:vegfc*^{SS}*-2Amcherry* as well as pCS*vegfc*^{SS}*-HA*.

Vegfc^{SS} secretion analysis in cultured mammalian cells.

293Ts cells cultured in DMEM supplemented with 10% calf serum were transiently transfected with either pCSzf*vegfc-HA* or *pCSzfvegfc*^{ss}*-HA*. At 72 hour post transfection, cells were collected and lysed in RIPA lysis buffer (RIPA-150mM NaCl, 50mM Tris ph 8.0, 1% NP-40, 0.1%SDS, Complete Mini (Roche)). Media was collected and concentrated using centrifugal filter units (Ultragel -10K, Millipore). Lysates and media were subjected to SDS-PAGE immunoblot analysis and probed with a monoclonal antibody recognizing the HA epitope (BABCO).

vegfc^{ss} rescue experiments

The pTol*fliebs:*vegfc^{SS}-2Amcherry was generated as described earlier (Chapter II, Methods). To assay rescue of the TD formation in *vegfc*^{um18} mutants by *vegfc*^{SS} we injected the embryos from an in-cross of Tg(*fli1a*:egfp)^{y1}*;vegfc*^{um18} heterozygous carriers with 25pg either of pTol2*fliebs*:vegfc-2Amcherry , pTol2*fliebs*:vegfc^{SS}-2Amcherry or pTol2*fliebs*:egfp-2Amcherry. In all cases, embryos were concomitantly injected with 25pg of *tol2 transposase* mRNA to facilitate genome integration. Injected embryos were allowed to develop until 5 dpf and subsequently selected for embryos expressing mCherry in endothelial cells. mCherry expressing embryos were then scored for presence of the TD and ultimately genotyped as described elsewhere. (Chapter III, Methods)

Riboprobes

Full length *furinA* was amplified using the following primers: forward 5’ GGGGACAAGTTTGTACAAAAAAGCAGGCTCCGCCACCATGGATCTCAGGCT TGCCTC’3 and reverse 5’

GGGGACCACTTTGTACAAGAAAGCTGGGTGGGGTTAAAGAGCACTTTGTGT TTTG'3, which were flanked by attB1 and attB2 site, respectively. p*furinA* was used as a template (Kimmel Lab). The resulting PCR product was used to generate pME*furinA* as described earlier. pME*furinA* was used to generate pCS*furinA* as described elsewhere (Chapter II, Gateway cloning). pCS*furinA* was linearized using *NotI* and purified as described elsewhere. T7 pol II (Roche) was used to generate antisense *furinA* probe (ref.). Standard whole-mount *in situ* hybridization was performed as described elsewhere [123].

Morpholino injections and Phenotyping

Embryos resulting from a Tg(*fli1a*:egfp)^{y1} in-cross were separately injected with 16ng standard control MO (Gene tools) and or 16ng *furinA SA9* MO [77](ref.). Injected embryos were subsequently assayed for general morphology and SeA length at 30 hpf as described elsewhere (Chapter IV). PLs formation was assessed in Tg(*fli1a:*egpf)^{y1} embryos at 2 dpf and formation of secondary intersomitic veins was assessed by assaying physiological venous or arterial flow through intersegmental vessels at 72 hpf (Chapter IV). Finally TD formation was assessed at 5 dpf. These phenotypic analyses were performed using a second antisense MO that was designed to target the splice acceptor sequence of the third exon of the *furinA* transcript. *furinASA3* MO.

Imaging of general morphology and circulation was performed using transmitted light on a Leica MZ FLIII using a Zeiss AxioCam MRc digital camera. Images of

blood vessels, PLs and TD in Tg(*fli1a*:egfp)^{y1} embryos were obtained using a Leica DMIRE2 confocal microscope (Objective: HC PL APO 20x/ 0.70CS).

Furin Inhibitor treatments

Wildtype Tg(*fli1a*:egfp)^{y1} embryos were treated in 8uM Furin I inhibitor (Cal Biochem) or 0.01% DMSO vehicle egg water solution at 30 hpf and allowed to develop and subsequently assessed TD formation as described above.

FurinA loss of function and Notch activation.

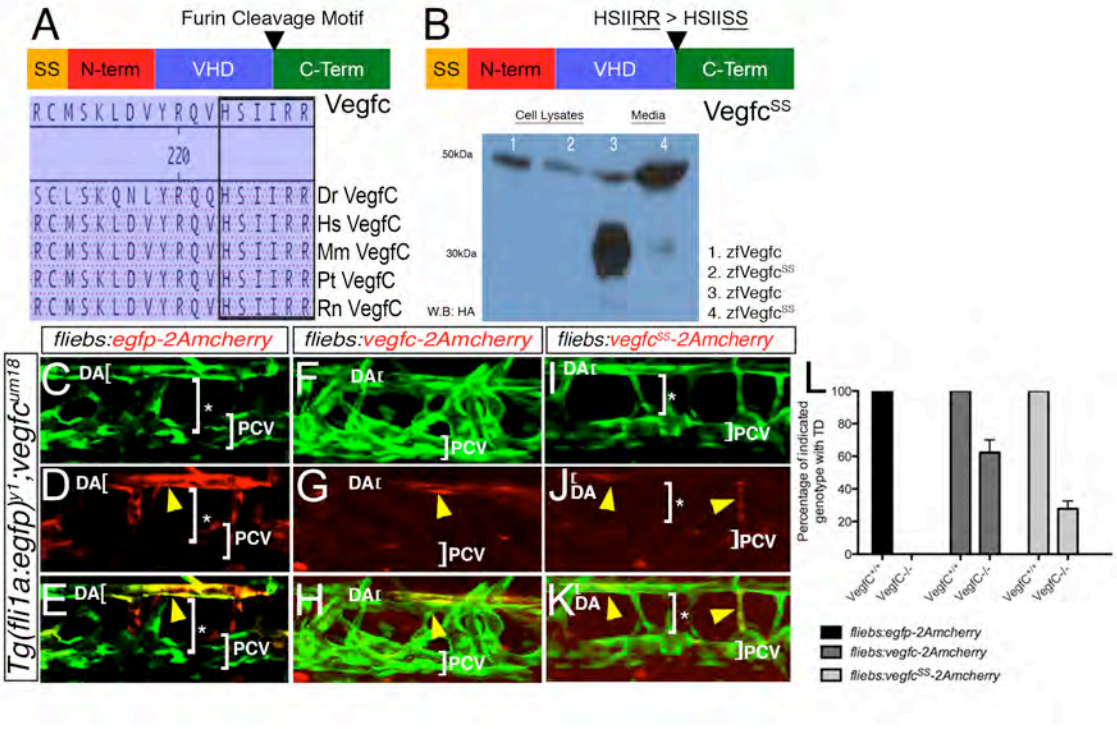
To assess Notch activity in *furinA* deficient embryos we separately injected (TgTP1:d2egfp^{um42};Tg:Fli1ep:dsRedEx^{um13}) embryos with 16ng of either *furinASA9* MO or standard control MO. Embryos were qualitatively assayed for differences in Notch activation in blood vessels as judged by a yellow fluorescence at 30 hpf.

Results

Cleavage at the C-terminal domain of Vegfc is required for lymphatic development in zebrafish.

Inspection of zebrafish Vegfc functional domains revealed a conserved Furin processing motif that separates the VHD from the C-terminal domain (Figure 1A). Interestingly, the *vegfc*^{um18} truncation allele eliminates this conserved Furin processing motif in Vegfc. As shown previously (Chapter III), embryos harboring this mutation fail to form lymphatics. Therefore, we determined the importance of this motif by assessing the ability of a cleavage-resistant form of Vegfc to rescue lymphatic defects in *vegfc*^{um18} mutants. For this purpose, we constructed a version of Vegfc containing amino acid substitutions in the furin cleavage motif (HSIIRR>HSIISS, referred hereafter as zfVegfc^{SS} Figure 1B). Immunoblot analysis of cell lysates expressing hemagglutinin epitope tagged zfVegfc^{SS} confirmed its inability to be cleaved (Figure 1B, compare lanes 1 and 2). Interestingly, we found significant amounts of uncleaved zfVegfc^{SS}-HA in the medium suggesting that furin-mediated cleavage is not required for Vegfc secretion (Figure 1B, lane 4).

To determine the functional effects of blocking furin-mediated cleavage, we drove expression of either wild type or cleavage-resistant *vegfc* (referred to as vegfc^{ss}) fused to 2A-cherry specifically in arterial endothelial cells of *vegfc*^{um18} mutant embryos. In control *vegfc*^{um18} embryos expressing egfp-2A-cherry in arterial endothelial cells, we noted full penetrance of lymphatic defects displayed by


A
Furin Cleavage Motif
SS
N-term
VHD
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Vegfc
R C M S K L D V Y R Q V H S I I R R
220
S C L S K Q N L Y R Q Q H S I I R R Dr VegfC
R C M S K L D V Y R Q V H S I I R R Hs VegfC
R C M S K L D V Y R Q V H S I I R R Mm VegfC
R C M S K L D V Y R Q V H S I I R R Pt VegfC
R C M S K L D V Y R Q V H S I I R R Rn VegfC
B
HSIIRR > HSIISS
SS
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VHD
C-Term
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50kDa
30kDa
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1. zfVegfc
2. zfVegfc^SS
3. zfVegfc
4. zfVegfc^SS
fliebs:egfp-2Amcherry
fliebs:vegfc-2Amcherry
fliebs:vegfc^SS-2Amcherry
Tg(fli1a:egfp)^y1;vegfc^um18
C DA[PCV
D DA[PCV
E DA[PCV
F DA[PCV
G DA[PCV
H DA[PCV
I DA[PCV
J DA[PCV
K DA[PCV
L
Percentage of indicated genotype with TD
100
80
60
40
20
0
VegfC+/+
VegfC-/-
VegfC+/+
VegfC-/-
VegfC+/+
VegfC-/-
fliebs:egfp-2Amcherry
fliebs:vegfc-2Amcherry
fliebs:vegfc^SS-2Amcherry

Figure 1.IV Cleavage of the Vegfc C-terminal domain of Vegfc is required during lymphatic development.

(A) Graphic describing Vegfc protein domains. SS-Signal Sequence(Orange). N-Terminal domain(Blue). VHD-VEGF Homology Domain (Red). C-Terminal Domain(Green). Black arrowhead represents Furin cleavage motif. Black box outlines the conserved (HSIIRR) Furin cleavage motif. **(B)** Graphic describing HSIIRR> HSII**SS** mutation. Biochemical evidence that a mutation in the Furin clevage domain of zebrafish Vegfc (zfVegfc^{SS}) inhibits processing. Cell lysates and Media from HEK293T cells transiently expressing zfVegfc-HA and zfVegfc^{SS}-HA separately, were used to monitor both zfVegfc and zfVegfc^{SS} biosynthesis and secretion. Cytoplasmic and Media fractions were collected 72 hrs post transfection and subsequently analyzed by immunoblot with HA epitope antisera. **(C-L)** Rescue experiment showing partial rescue of thoracic duct formation in *vegfc^{um18}* mutants by arterial expression of *vegfc^{SS}-2Amcherry*. (C-K). Confocal micrographs of trunk blood vessels at 5 dpf. Anterior is to the left dorsal is up. DA, Dorsal aorta. PCV. Posterior Cardinal Vein. TD. Thoracic Duct. (C-E) Tg*(fli1a:egfp)^{y1}vegfc^{um18}* mutant injected with 25pg of Tol2 transposase and pTol2egfp-2Amcherry. Yellow arrowheads indicate coloaclization of Egfp and mCherry. Bracket with asterisk indicate absence of TD. (F-H) Tg(*fli1a:egfp)^{y1}vegfc^{um18}* mutant injected with 25pg of Tol2 transposase and pTol2vegfc-2Amcherry. Yellow arrowheads indicate coloaclization of Egfp and mCherry. White arrows indicate mosaic rescue of the TD. (I-K) Tg*(fli1a:egfp)^{y1}vegfc^{um18}* mutant injected with 25pg of Tol2 transposase and pTol2vegfc^{um18}-2Amcherry. Yellow arrowheads indicate coloaclization of Egfp and mCherry. Bracket with asterisk indicate absence of TD. (L) Quantification of rescue. Expressed as the percent of indicated genotype of an injected clutch of *um18* heterozygous carriers. NOTE: Portions of this figure appear in Chapter III

vegfc^{um18} mutants, while expression of *fliebs:vegfc-P2Amcherry* rescued TD formation in greater than 60 percent of mutant embryos (Figure 1C-H,L Note: this data also seen in Chapter II). By contrast, arterial cells expressing of *fliebs:vegfc*^{SS}*-P2Amcherry* was able to partially rescue TD formation in *vegfc*^{um18} (Figure 1I-L), although the degree of rescue in this case was significantly lower compared to that of *fliebs:vegfc-2A-mcherry* (Figure 3L). Taken together, these data suggest that furin-mediated cleavage of Vegfc may be required for proper lymphatic patterning in zebrafish.

Loss of *furinA* function in zebrafish suggests a role during lymphatic vessel formation.

Given the decreased ability of a furin-resistant form of Vegfc to rescue lymphatic defects in *vegfc*^{um18} mutants, we investigated whether Furin itself was required for lymphatic development. While the zebrafish genome encodes two duplicates of the furin gene (*furina* and *furinb*) the expression pattern of *furina* suggested a possible role in lymphatic patterning. By whole mount *in situ* hybridization analysis, we observed *furinA* expression in neuromasts and cells dispersed along the lateral line at 2 dpf (Figure 2A). These structures, which can be visualized along with the developing vascular and lymphatic vessels in Tg(fli1ep:dsredEx;Nfkb1:egfp) double transgenic embryos, are located immediately adjacent to the PL as it is forming (Figure 2B-C). To investigate if *furinA* was required for lymphatic vessel development, we injected *Tg(fli1a:egfp)*^{y1} embryos with a previously published antisense morpholino

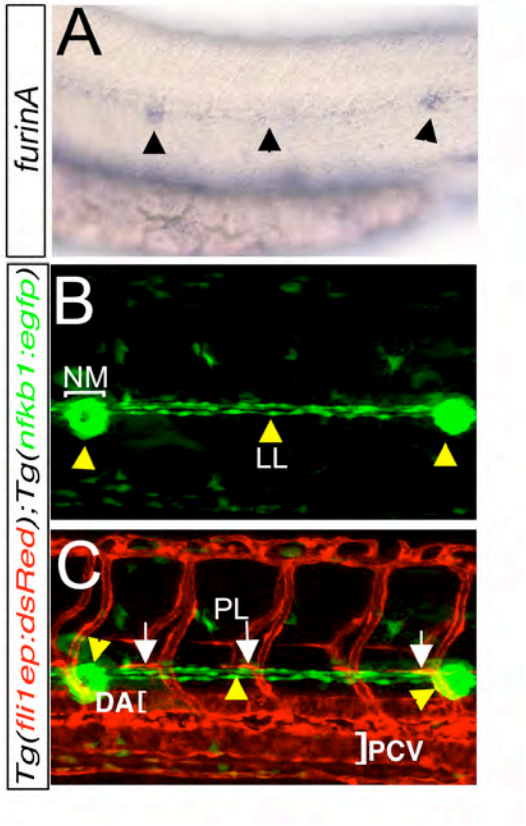


Figure 2.IV Sites of Furin expression are adjacent to developing parachordal lymphangioblasts.

(A-C) micrographs 2 dpf zebrafish embryos. Anterior is to the left dorsal is up. DA, Dorsal aorta. PCV. Posterior Cardinal Vein. TD. Thoracic Duct. NM-Nuero Masts. LL. Lateral Line. PL. Parachordal Lymphangioblasts. (A) Whole mount *in situ* hybridization for *furinA.* Black arrowheads indicate expression in the LL and NM. (B-C) Confocal micrographs of trunk blood vessels at 2 dpf. in Tg(fli1ep:dsredEx;Nfkb1:egfp) double transgenic. (B) Yellow arrowheads highlight similar trangene expression to that of *furinA* in the LL. Brackets indicate transgene expression in the (C) White arrows depict PL formation adjacent to the LL and NM.

NM.

targeting the *furinA* transcript. *furinA*-deficient embryos displayed relatively normal morphology when compared to control morphant siblings at 30 hpf, although slight curvature of the tail was noted (Figure 3A-B). Formation of the primary circulatory network was not affected by loss of *furinA* (Figure 3C-D). However, at 5 dpf *furinA*-deficient embryos displayed notable defects in TD formation when compared to control morphant siblings (Figure 3E-G). To investigate whether loss of TD in *furinA* morphants was due to earlier defects in PL formation, we scored the formation of these structures in *Tg(fli1a:egfp)*^{y1} Ctrl MO and *furinA* MO injected embryos. We observed fewer PLs in *furinA* defiecient embryos when compared to control morphant siblings (Figure 4A-C), while secondary intersomitc vessels appeared normal (Figure 4D). Additionally, we found that a second non-overlapping morpholino targeting the *furinA* transcript showed similar effects on lymphatic patterning (personal observation).

Our results suggest that *furinA* is required for proper sprouting of PLs and subsequent pattering of lymphatics in zebrafish. To determine whether defects observed in lymphatic patterning were specific to Furin activity during this process we pharmacologically inhibited its function using Furin inhibitor I, a catalytic inhibitor of Furin(ref.). Tg(fli1:egfp)^{y1} embryos were treated with Furin inhibitor I at 30 hpf, a time prior to PL sprouting and subsequently assayed for the TD formation at 5 dpf. Tg(fli1:egfp)^{y1} embryos treated with Furin inhibitor I displayed a significant loss of TD formation at 5 dpf when compared to vehicle

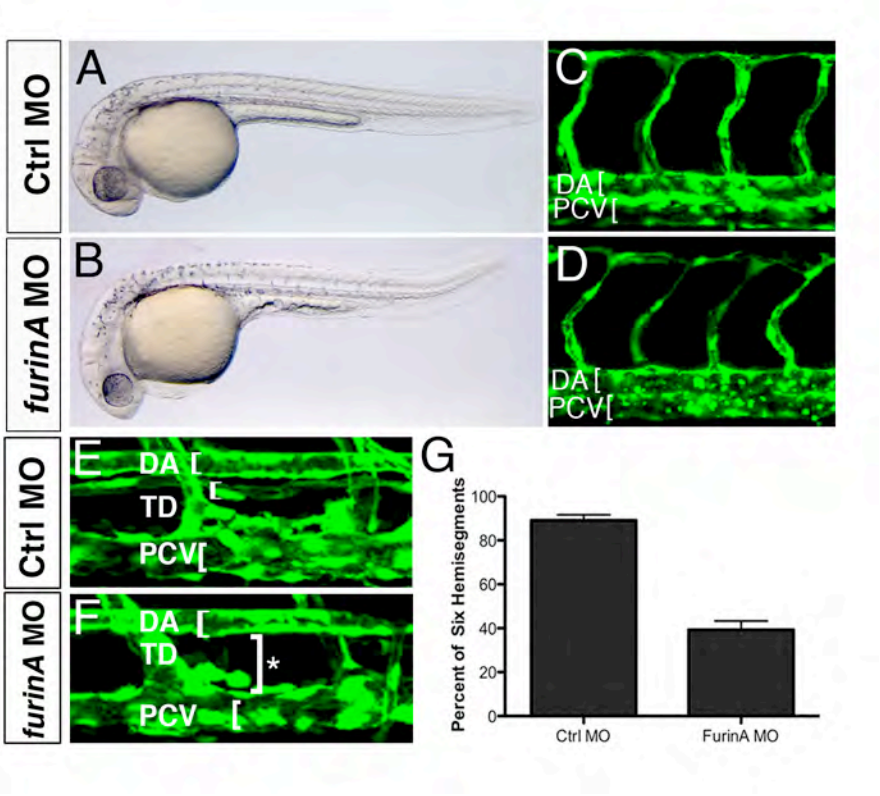


Figure 3.IV *furinA* is required for lymphatic vessel formation in zebrafish.

(A-B) Bright field illumination of 30 hpf Tg*(fli1a:egfp)*^{y1} zebrafish embryos. Anterior is to the left dorsal is up. (A) Tg*(fli1a:egfp)*^{y1} embryo injected with Ctrl MO. (B) Tg*(fli1a:egfp)*^{y1} embryo injected with *furinA* MO. **(C-D)** Confocal micrographs 30 hpf. zebrafish embryos. DA, Dorsal aorta. PCV. Posterior Cardinal Vein. (C) Tg*(fli1a:egfp)*^{y1} embryo inject with Ctrl MO. (D) Tg*(fli1a:egfp)*^{y1} embryo injected with *furinA* MO. **(E-F)** Confocal micrographs 5 dpf. zebrafish embryos. DA, Dorsal aorta. PCV. Posterior Cardinal Vein. TD. Thoracic duct. (E) Tg*(fli1a:egfp)*^{y1} embryo inject with Ctrl MO. (F). Tg*(fli1a:egfp)*^{y1} embryo injected with *furinA* MO. Bracket with asterisk denotes albescence of TD. **(G)** Quantification of TD in embryos. Embryos were assayed for coverage of TD between six hemisegments of the trunk. Values shown are based on the average of three independent experiments.

treated controls (Figure 4E). These data further suggest a requirement for FurinA function during the lymphatic patterning process.

In addition to our proposed role for Furin in Vegfc activation, Furin function is also required for Notch activation[124](ref). Recently, studies have shown the requirement Notch signaling during lymphatic vessel development[125]. To address any indirect effects on lymphatic development by loss of Notch signaling, we used a double transgenic line (Tg*Tp1*:d2egfp^{um42};Tg:*fli1ep*:dsRedEx^{um13}) that allowed visualization of dynamic Notch activity (Tg*Tp1*:d2egfp) as well as blood vessel formation (Tg:*fli1ep*:dsRedEx) in *furinA* deficient embryos. Evaluation of blood vessel formation at 30 hpf in *furinA* deficient embryos reveals levels of Notch activation that where qualitatively indistinguishable from control morphant embryos (Figure 5A-B). Taken together, these data suggest that FurinA itself may be responsible for the C-terminal cleavage of Vegfc during lymphatic vessel development in zebrafish.

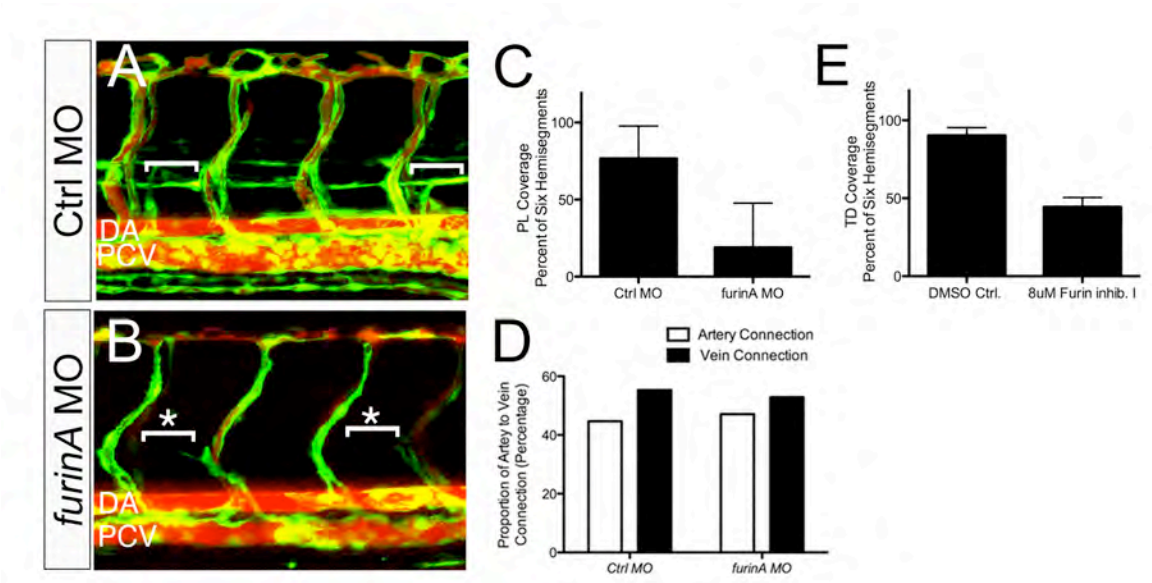


Figure 4.IV *furinA* deficiency negatively affects formation parachordal lymphangioblasts.

(A-B) Confocal micrographs of trunk blood vessels at 2 dpf. Anterior is to the left dorsal is up. DA, Dorsal aorta. PCV. Posterior Cardinal Vein. Parachoradal lymphangioblast (PL). Embryos were subjected to microangiography. (A) Tg*(fli1a:egfp)*^{y1} embryo inject with Ctrl MO. Brackets indicate PL formation (F). Tg*(fli1a:egfp)*^{y1} embryo injected with *furinA* MO. Brackets with asterisks denote absence of PLs. **(C)** Quantification of PLs in embryos. Embryos were assayed for coverage of PLs between six hemisegments of the trunk. **(D)** Quantification of percent artery and vein connections in Ctrl MO and *furinA* MO injected embryos. **(E)** Quantification of TD in embryos embryos treated with 8uM Furin inhibitor I at 2 dpf . Embryos were assayed for coverage of TD between six hemisegments of the trunk at 5 dpf. Values shown are based on the average of three independent experiments.

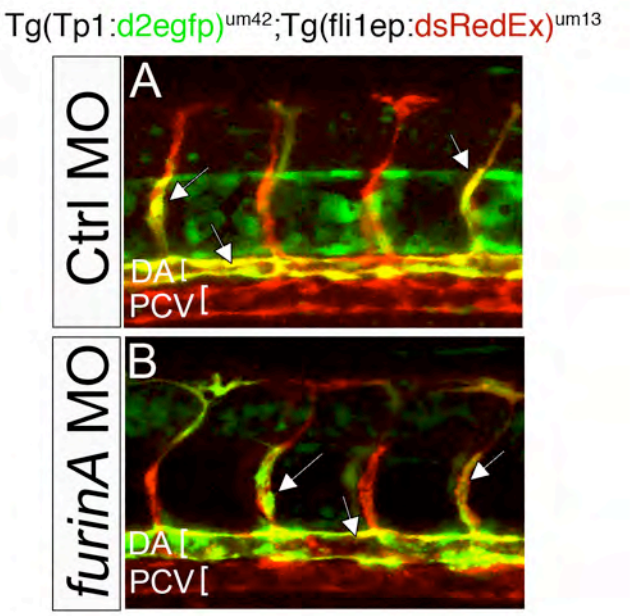


Figure 5.IV Notch signaling in blood vessels is unaffected by *furinA* deficient embryos.

(A-B) Confocal micrographs of trunk blood vessels at 30 hpf in Tg*Tp1*:d2egfp^{um42};Tg:*fli1ep*:dsRedEx^{um13} zebrafish embryos. Anterior is to the left dorsal is up. DA, Dorsal aorta. PCV. Posterior Cardinal Vein. (A) Ctrl MO injected embryo. White arrows denote Notch activation in blood vessels (yellow). (B) *furinA* MO injected embryos. White arrows indicate Notch activation in blood vessels.

Discussion

In this chapter we present genetic evidence linking Furin function to the C-terminal domain processing of Vegfc during lymphatic development in zebrafish. These conclusions are borne out of our findings, which demonstrated that a non-cleavable form of Vegfc (*vegfc*^{SS}) only partially rescued lymphatic pattering in *vegfc*^{um18} mutant embryos. Our biochemical studies suggest that cleavage at the C-terminal domain of Vegfc is not a pre-requisite for its secretion and that perhaps some other aspect of its function was affected. One possibility may be an inability to activate Flt4. Indeed, biochemical analyses of Vegfc function have found that a non-cleavable mutant of Vegfc, similar to the one presented in this study, is able to bind and activate Flt4 albeit to lower levels than that of mature wild type Vegfc[60]. This decreased activation may explain why *vegfc*^{SS} was able to partially rescue lymphatics in *vegfc*^{um18} mutant embryos. Together, these data suggest that complete processing of Vegfc is required to fully induce Flt4 activity during lymphatic vessel formation in zebrafish.

Our finding that Furin mediated C-terminal processing of Vegfc was required for optimal lymphatic patterning led us to investigate a role of Furin itself during vascular development. Our results suggest that *furinA* is required during lymphatic development perhaps by coordinating the sprouting of lymphatic progenitors. These findings were further supported by our pharmacological studies, which demonstrated that inhibition of Furin function during this process ultimately effected the formation of the TD. These observations were reminiscent

of *vegfc* and *flt4* deficiency[15,17,33], suggesting a link between FurinA activity and that of Vegfc signaling during lymphatic patterning. Studies in zebrafish have found that sprouting and subsequent migration of lymphatics is quite complex [36,125,126]. During this process, cells need to sprout off the PCV arrest the horizontal myoseptum and subsequent migrate ventrally to be come part of the TD; exactly what aspect of this process is affected by *furinA* deficiency will likely require further cell biological investigation.

Previous studies in cultured cells have found that Vegfc is a substrate for Furin[74]. However, the exact mechanism of how Furin exerts its effects on Vegfc *in vivo*, remain unclear. Previous studies have found that PLs normally migrate along arteries during lymphatic development[36]. Additionally, we have shown that *vegfc* is expressed in arterial endothelial cells[14] from where it is produced in order to pattern lymphatics. Our *in situ* expression analysis demonstrated that *furinA* is expressed in cells of the lateral line, which are in close proximity to developing PLs. Thus it is plausible that Furin may process arterial derived Vegfc in a non-cell autonomous manner during lymphatic development. Two lines of evidence suggest that this may be the case. First, cell biological studies have found that Furin is transmembrane protein that cycles between the TGN the cell membrane, suggesting it can act at the cell membrane [120,122]. Second, during the course of our biochemical analysis we observed that cell lysates from mammalian cells expressing Vegfc contained little to no evidence of processed Vegfc (Figure 1B lane 1), suggesting processing of Vegfc occurred out side the producing cell and

further implicating the C-terminal domain of Vegfc during its secretion. While this mechanism of action may hold true, further *in vivo* molecular analysis of the expressed processed forms of Vegfc during vascular development should shed further light on this process.

Interestingly, we found that loss of *furinA* had no effect on the formation of segmental arteries or that of secondary intersomitic vessels, which are affected by deficiencies in Vegfc/Flt4 signaling [15,34]. This difference in phenotype suggests the intriguing possibility that differential processing may regulate the diverse aspects of Vegfc signaling. Thus, while complete processing of Vegfc may be required for its function during lymphatic development, similar processing could be dispensable for its function during segmental artery and intersomitic vessel development. As a result, Furin mediated processing may induce context dependent effects of Vegfc during the formation of these vessel types. However, the fact that we do not see any effects on segmental artery or intersomitic vessel formation may also suggest the existence of another propeptide convertase that cleaves Vegfc during the formation of these two vessel types. Indeed, studies have found that Vegfc can be a substrate for related propeptide convertases such as PC5 and PC7[74].

Our studies imply that Vegfc is the major substrate for FurinA during lymphatic vessel development in zebrafish. As a result, we find that loss of *furinA* does not affect processing of Notch, implying that the observed lymphatic defects are probably due to its effects on Vegfc signaling. In addition to Notch, studies have

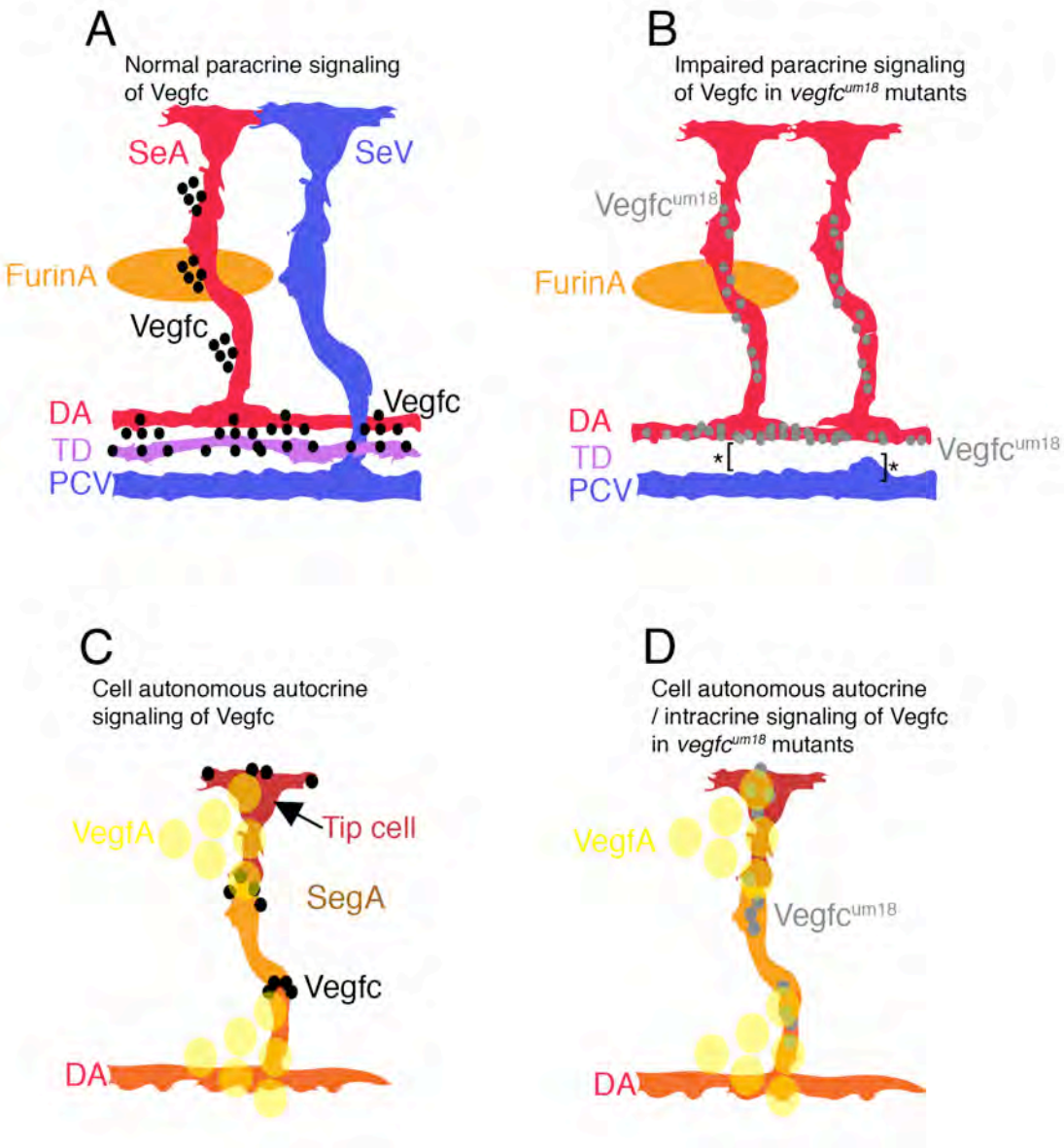
found that Sema3E is substrate of Furin[127-129]. Recently, studies in zebrafish have described a role for Sema3E signaling during developmental angiogenesis[127]. We find that the primary angiogenic network forms normally in *furinA* deficient embryos, suggesting that Sema3E is not a substrate for FurinA during this process and would not affect lymphatic patterning.

In conclusion, the data presented here suggest a functional link between Furin and C-terminal cleavage of Vegfc during lymphatic vessel development. Moreover, they suggest the intriguing possibility that differential processing of Vegfc may regulate its diverse effects during vascular development. However, the question of where this processing occurs and the exact cell biological effect of *furinA* deficiency on PLs during lymphatic vessel development will require further investigation.

FINAL THOUGHTS AND FUTURE DIRECTIONS

Previous studies have demonstrated that Vegfc/Flt4 signaling is essential for both blood and lymphatic vessel development in vertebrates [14-16,62]. However, the molecular mechanism(s) of how it orchestrates their formation remains unclear. In this study we used a transgenic haploid screen to identify genes affecting Vegfc/Flt4 signaling. We identified and cloned a truncation allele of *vegfc* (*vegfc*^{um18}), which completely lacks the Vegfc C-terminal silk homology domain. These mutants display defects in vein and lymphatic vessel formation but normal developmental angiogenesis (Chapter II). Characterization of this allele led to the finding that the primary defect in *vegfc*^{um18} mutants was a general failure in vein and lymphatic vessel sprouting, which was rescued by transgenic expression of wild type full length *vegfc* in the dorsal aorta (DA) (Chapter II). Further genetic and biochemical analysis of *vegfc*^{um18} revealed profound paracrine signaling defects, which likely result in the observed loss of lymphatic and venous structures in these mutants. Together, our findings imply that paracrine Vegfc derived from arteries acts as a chemo-attractant for lymphatic endothelial cells during lymphatic vessel formation (Figure 1A and Chapter III). Furthermore, they suggest that the C-terminal domain is absolutely required for efficient paracrine activity of Vegfc in this context vascular development (Figure 1B and Chapter III). Interestingly inefficient secretion of Vegfc in *vegfc*^{um18} mutants did not negatively affect SegA formation. Genetic and molecular analysis showed that normal SeA formation in these mutants was possibly due to functional redundancy by the Vegfa signaling pathway and weak autocrine and/or intracrine signaling by

Vegfc^{um18} in the context of SeA formation. Additionally, we observed that *vegfc* was required in an endothelial cell autonomous manner for tip cell occupancy, together implying a cell autonomous autocrine role for Vegfc during developmental angiogenesis (Figure 1 C-D and Chapter III). Finally, we present genetic evidence that links processing of the C-terminal domain of Vegfc by FurinA during lymphatic development (Figure 1A and Chapter IV). However, *furinA* appears to be dispensable during blood vessel development (Chapter IV). Taken as whole, the data presented in this thesis demonstrate two discrete modes of signaling by Vegfc/Flt4 during blood and lymphatic vessel development in zebrafish. They imply that context dependent regulation of Vegfc secretion and processing play a pivotal role in the how these vessels are formed in zebrafish.


A
Normal paracrine signaling of Vegfc
SeA
SeV
FurinA
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Vegfc
DA
TD
PCV
B
Impaired paracrine signaling of Vegfc in vegfc^um18 mutants
Vegfc^um18
FurinA
DA
TD
PCV
Vegfc^um18
C
Cell autonomous autocrine signaling of Vegfc
VegfA
Tip cell
SegA
Vegfc
DA
D
Cell autonomous autocrine / intracrine signaling of Vegfc in vegfc^um18 mutants
VegfA
Vegfc^um18
DA

Figure 1. Model. Characterization of vegfcum18 reveals context dependent signaling mechanisms during blood and lymphatic vessel development in zebrafish.

(A-C) Anterior is to the left dorsal is up. DA, Dorsal Aorta. PCV, Posterior Cardinal Vein. TD, Thoracic Duct. SeA, Segmental Artery. SeV, Segmental Vein. Bracket and asterisk denote absence of TD and SeV. **(A-B)** Graphic describing the role of Vegfc during lymphatic vessel development in zebrafish. (A) During normal lymphatic vessel development, paracrine signaling by Vegfc from the DA and SegA guide lymphatic endothelial cells to ultimately form the TD. FurinA expression at the lateral line is required for processing Vegfc, for a yet unknown aspect of parachordal lymphangioblast (PL) formation. (B) Absence of the C-terminal domain in *vegfc*^{um18} mutants impairs Vegfc paracrine activity, which leads to a failure of SeV and PLs sprouting and ultimate loss of the TD. **(C-D)** Graphic describing the role of Vegfc during developmental angiogenesis in zebrafish. (C) During normal SeA formation, cell autonomous autocrine signaling by Vegfc promotes angiogenesis at the leading tip cell. Additionally, formation of SeA requires inputs from the VegfA signaling pathway, where *vegfa* is supplied by adjacent somites in a paracrine manner. (D) Absence of the C-terminal domain in *vegfc*^{um18} mutants has no effects on SeA formation. This is likely due to redundant signaling from VegfA signaling pathway coupled to functional autocrine and/or intracrine signaling by Vegfc^{um18} within SeA themselves.

What does vegfc^{um18} tell us about Vegfc function during lymphatic development?

In this study we show that the C-terminal domain of Vegfc is required for its paracrine effects by coordinating PL and secondary intersomitic vessel formation. One question that arises is whether the C-terminal domain of Vegfc is a general factor that increases secretion. Further insight to this question may come from rescue experiments to investigate whether expression of the C-terminal domain on its own can recue Vegfc^{um18} defects. This can be performed both *in vivo* and *in vitro,* by assessing whether the Vegfc C-terminal domain can rescue the TD in *vegfc^{um18}* mutant embryos and secretion in mammalian cells expressing VEGF-CΔC.

During the course of our study we noted that heterozygous *vegfc^{um18}* embryos displayed defects in lymphatic development. Interestingly, formation secondary intersegmental vessels were unaffected in these embryos, implying that higher levels of Vegfc/Flt4 signaling are required during lymphatic vessel development. These findings coincide with a recent study, which demonstrated that a hypomorphic allele of *flt4* in mouse displayed severe defects in lymphatic vessel formation although blood vessel development was unaffected, strikingly similar to *vegfc^{um18}* [63]. Moreover, a kinase dead allele of *flt4* in zebrafish also displays profound defects in lymphatic vessel formation, while developmental angiogenesis is unaffected [15]. Together these findings suggest that distinct levels of Vegfc/Flt4 signaling may be required for separate formation of vessel types. Further insight into this hypothesis may come from studies that evaluate the

formation of distinct vessel types by varying levels of Vegfc/Flt4 signaling using MOs targeting their expression. This study could be complemented by the assessing the formation of distinct vessels in separate alleles of either of these genes.

Finally, several lines of evidence from our studies suggest that there may be a context dependant role for Vegfc during lymphatic development. First, we find that Vegfc/Flt4 signaling is required throughout lymphatic development, suggesting this pathway maybe used in the migration of PLs to form the TD. Second, we find that cleavage of the Vegfc C-terminal domain is required for lymphatic development but not its secretion. Third, we present expression and functional data that links FurinA to Vegfc processing in perhaps a non-cell autonomous manner at the horizontal myoseptum. Interestingly, *vegfc*^{um18} mutants fail to form intersegmental vessels while *furinA* deficient embryos seem to be normal in this respect implying that perhaps Furin activity is independent of venous sprouting. These data imply that FurinA may be involved in some other aspect of lymphatic development, perhaps guidance, by enacting a context dependent cleavage of Vegfc. To investigate whether this is the case, we first have to pinpoint the exact cellular defect in PLs of *furinA* deficient embryos, which can be done using current tools available. These include time-lapse microscopy and the use transgenic and mutant lines, which should allow a better assessment of PL formation in *furinA* deficient embryos. In addition to this, generation of antibodies that recognize distinct forms of Vegfc could also be used

to elucidate this proposed context dependent function of Vegfc. Such antibodies would allow direct whole-mount visualization of the distinct processed forms of Vegfc. However, such antibodies are currently unavailable.

While the aforementioned scenario may be plausible, the existence of other signaling pathways may also be involved in the guidance of lymphatics. During our study we observed that chemical inhibition of Flt4 during lymphatic development had incomplete penetrant effects on TD formation, implying functional redundancy by other signaling pathways. In fact, molecules involved in neuronal pathfinding have also been implicated in vascular development. In particular, a recent study demonstrated that the canonical neuronal guidance factor, *unc5b*, was necessary for the formation of PLs in zebrafish [130]. Therefore, it is plausible that inputs from the Vegfc/Flt4 signaling pathway along with those involved in neuronal pathfinding are required for proper guidance of lymphatic progenitors during lymphatic vessel formation.

What role does Vegfc play during blood vessel development?

Previous studies in our lab demonstrated that embryos deficient for *vegfc* displayed partial formation of SegA [14]. In our study, we find that blood vessel defects in *vegfc*^{um18} embryos are masked by inputs from the Vegfa signaling pathway further implying a role for Vegfc during SegA formation. Interestingly, studies have found that Vegfc largely induces the formation of Vegfr2/Vegfr3 heterodimers [56] and that the formation of these heterodimers is required for sprouting and migration of blood vessel endothelial cells. Furthermore, blocking

heterodimer formation decreased *in vivo* vascular network formation in NOD-SCID mice xenograted with Vegfc matrigel plugs containing human blood vessels [56,106]. Taken together these data suggest that Vegfc induced formation of Vegfr2/Vegfr3 hetrodimers may be required for sprouting and migration during active angiogenesis. Further insight in to the exact cellular role for Vegfc during blood vessel development may come from time-lapse imaging of migration and proliferation events in the SegA of *vegfc* deficient zebrafish embryos in a blood vessel transgenic background.

We demonstrated that deficiencies in Vegfa and Vegfc signaling severely affected sprouting of SegA. Thus one question that arises is: how do endothelial cells coordinate sprouting? During sprouting angiogenesis a subset endothelial cells, tip cells, need to be selected for sprouting. Selected cells undergo phenotypic changes that allow them to acquire invasive motile behavior. Vegfa signaling has been shown to involved in the selection and guidance of tip cells, in addition to its role in proliferation during angiogenesis [31,78]. Given the diverse functions of Vegfa signaling during the sprouting process, how is it that tip cells maintain their polarity and continual directional migration? Until now the cell autonomous function of Vegfc during blood vessel development was unknown. Our mosaic analyses suggest that Vegfc plays a role in defining tip cell behavior. This is consistent with the observation that *vegfc* is concomitantly expressed with Vegfr3 and Vegfr2, at the leading tip cells of angiogenic sprouts [62]. Moreover, Vegfc induced Flt4/Vegfr2 heterodimers preferably localized to the tip cell

position [56]. Together, these observations suggest that Vegfc is required to in an endothelial cell autonomous manner for a yet unknown aspect of tip cell physiology. However, based on our findings and those by others we can speculate that Vegfc may act in a cell autonomous manner to induce Vegfr3/Vegfr2 heterodimers to elicit a signal that results in the invasive and directional migratory phenotype exhibited by tip cells during angiogenesis.

Limitations and future directions for the forward genetic screen presented here.

Our study demonstrates that screening for early defects in PHBC formation in mutant transgenic haploids can be used as an indirect way to find genes involved in vein and lymphatic vessel formation. Although we were able to recover a mutant using this strategy, a variety of points may have limited our study. The first of them being the amount of genomes scored. During this screen we scored a total of 410 haploid genomes, which is about one-third of the amount of genomes screened in our previous haploid screen[45] and roughly a tenth of the original genomes scored in the zebrafish F3 screens performed in Tubingen and Boston[92,93]. Thus, increasing the size of the scored genomes may have led to the recovery of more mutants in this screen. In addition to this limitation, we also encountered a problem in the generation of F2 families (Table 1, Chapter III). Of the four putant F1 females with observed PHBC defects in their haploid progeny, we were only able to recover two that could be out crossed to produce an F2 generation. Unfortunately, we were only able to produce one F2 generation. This low recovery of F2 families may be attributable to technical difficulties involving

the injury to the F1 female as a result of multiple *in vitro* fertilization attempts, which could have lead to problems in fecundity (personal observation).

Screening for early defects in vein formation as an indirect way to identify lymphatic mutants could fail to identify mutations affecting specific aspects of lymphatic vessel formation that are independent of vein morphogenesis. One example would be genes required for specification of lymphatic endothelia cells. Studies have demonstrated that specification of lymphatic endothelial cells is independent of Vegfc/Flt4 signaling and occurs prior to their sprouting, which does requires Vegfc/Flt4 signaling [16,33]. Thus, we would potentially fail to identify mutant alleles of factors such as *sox18 and prox1*, both of which are essential for lymphatic specification[37,38]. Importantly, deficiency in these genes has no effect on vein morphogenesis (personal observation). Very little is known about the genes required for lymphatic vessel specification, therefore further insight may come from screens designed to target aspects of *sox18* and/or *prox1* function.

Using vegfc^{um18} as a tool for future forward genetic screens.

vegfc^{um18} allele displays phenotypic advantages that could be used as way to screen for genes involved in distinct aspects of blood vessel development. First, we noted that heterozygous *vegfc^{um18}* embryos displayed loss of PL sprouting and subsequent TD, however sprouting of secondary intersomitic vessels and PHBC were unaffected, highlighting the distinct requirement for Vegfc signaling during these processes. These discrete phenotypic outputs could be taken advantage of in order to carry out an enhancer screen [131]. For example, one

could cross the F1 progeny, derived from mutagenized males, into the sensitized *vegfc*^{um18} heterozygous background and subsequently screen F3 progeny for separate defects in secondary intersomitic vessels or PHBC. One could also screen for mutants that affected both, although this last phenotypic method of screening could lead to the identification of another *vegfc* allele. Nevertheless, performing an enhancer screen in the *vegfc*^{um18} sensitized background could lead to the identification of novel alleles that modulate the formation of distinct blood vessels.

During the course of this study we also noted that we were sometimes able to recover adult homozygous *vegfc*^{um18} mutants. These mutants displayed defects associated with the inability to drain fluid, similar to patients with lymphedema[10]. Assuming you were able to breed these fish, one could envision performing a suppressor haploid screen to identify mutants that suppressed the PHBC phenotype. For example, one could cross the F1 progeny (derived from mutagenized homozygous *vegfc*^{um18} males) to homozygous *vegfc*^{um18} females to generate an F2 family of homozygous *vegfc*^{um18} fish carrying germline mutations. Haploid embryos would be generated from F2 females and subsequently screened for PHBC formation. Embryos carrying a suppressor mutation would result in 50% of the progeny rescuing PHBC formation and 50% displaying complete penetrance of the *vegfc*^{um18} mutation. This screening approach could result in the identification of genes with possible disease relevance.

Finally, one could also envision a third type of screen using adult homozygous *vegfc*^{um18} mutants that involves pharmacological compounds. As mentioned earlier, adult homozygous *vegfc*^{um18} mutants that make it to adulthood display overt lymphatic defects. Given the availability of affordable pharmacological libraries it would be feasible to screen a library of compounds for agents that ameliorated lymphatic defects in adult homozygous *vegfc*^{um18} mutants. As a result, this could lead to the identification of drugs that could potential be used to treat patients with congenital lymphedema.

APPENDICES

APPENDIX I

SPATIAL AND TEMPORAL OVEREXPRESSION OF VEGFC INDUCES PRO-ANGIOGENIC EFFECTS ON BLOOD VESSELS DURING DEVELOPMENTAL ANGIOGENESIS IN ZEBRAFISH.

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Dev. Dyn. 2007 vol. 236 (11) pp. 3077-87

Introduction

Vascular endothelial growth factor C (Vegfc) is a secreted factor that is involved in blood and lymphatic vessel development in vertebrates[8,14,16,59,64,84,132]. In zebrafish, *vegfc* is expressed in the developing blood vessels[14]. Unlike Vegf, Vegfc is proteolytically cleaved in order to be active[60]. Vegfc induces the phosphorylation of its endothelial cell specific receptor, Flt4. During embryonic development in zebrafish, *flt4* is initially expressed in the axial vasculature and developing segmental arteries (SeA) and later restricted to the posterior cardinal vein (PCV)[28]. Consistent with their expression, *vegfc* and *flt4* are required for proper blood and lymphatic vessel patterning in vertebrates [14,15,33,62].

The functional role of Vegfc in vascular development has mainly been attributed to lymphangiogenesis, even though its expression is evident during embryonic blood vessel development[14,62]. Overexpression of Vegfc in skin, muscle and mesenteric tissues of mice using adenoviral gene transfer noted strong induction lymphangiogenesis as well as effects on blood vessel morphology[132]. By contrast, a transgenic mouse allowing temporal control of endothelial produced Vegfc displayed potent angiogenic effects when expressed early in development [84]. In zebrafish, ectopic expression of *vegfc* mRNA causes mild aberrant sprouting of segmental arteries (SeA), indicating it has an effect on blood vessels[15]. Although ectopic mRNA expression is a rapid way the assess gene function in zebrafish, investigating the function of a gene with a highly restricted

expression pattern such as *vegfc*, requires the use of functional tools that allow spatial and temporal control of gene expression.

Engineering a system that allows spatial and temporal control of *vegfc* during embryonic development in zebrafish requires *1) an efficient way to test different epitope tags to monitor vegfc expression.* Vegfc is a secreted protein with a signal sequence. Therefore, testing various methods of tagging would be needed to assess proper functionality. In addition to a functional tagged Vegfc, it is necessary *to 2) express vegfc in either a temporal or endothelial cell autonomous manner*, which requires the use of distinct promoters. Lastly, this would also require a transgenic system that *3) allows low mosaic expression of a transgene, thus enabling immediate assessment of vegfc overexpression* without the generation of transgenic lines, which may take up to six months.

It is often the case that establishment of functional tools such as the one described above, results in laborious cloning efforts. Therefore, the availability of an efficient cloning system would simplify standard day-to-day cloning efforts, making the analysis of gene function in zebrafish less labor-intensive. Recently, a recombination-based cloning technique (Gateway Cloning) that allows efficient and rapid transfer of DNA fragments between plasmids, has been used to generate clone collections of both open reading frames (ORFs) and promoters that can be used together to rapidly generate constructs for transgenesis in *C. elegans*[133-135]. An important benefit of the Gateway system is its use to

simultaneously clone multiple contiguous DNA fragments within a single vector[134].

In this supplementary chapter, we establish a robust transgenic approach to show that Vegfc induces angiogenic effects through its receptor Flt4, when expressed in a temporal and endothelial cell-autonomous manner in zebrafish. To investigate the spatial and temporal effects of Vegfc on blood vessels, we adapt and engineer Gateway cassettes into vectors that are commonly used to assess gene function in zebrafish. This system enabled rapid generation and testing of vectors that allow controlled expression of a tagged ORF. We establish proof of concept for this system and build constructs that allow spatial and temporal control of Vegfc. We find that misexpression of Vegfc during blood vessel development induces ectopic angiogenesis in a *flt4*-dependent manner. Moreover, forced expression of Vegfc in endothelial cells induced similar angiogenic effects. These data suggest a pro-angiogenic role for Vegfc during developmental angiogenesis in zebrafish.

Materials and Methods

Zebrafish Lines and Maintenance

Zebrafish lines were maintained as described elsewhere[136]. Wild type EK (Ekkwill Farm derived), Golden (*gol*^{b1}) or *Tg(fli1a:egfp)*^{y1} embryos were used for injections.

Plasmid Construction

Generation of Entry Clones. The pDONR221, pDONRP2r-P3 and pDONRP4P1R were obtained as part of the Multisite gateway cloning kit (Invitrogen). pDONR plasmids were maintained in *ccdB*-tolerant bacteria (DB3.1) and grown in the presence of kanamycin and chloramphenicol. To generate an Egfp clone (pMEegfp3) to test C-terminal fusions, we amplified the Egfp CDS with the following primers Forward *attB1* 5'GGGGACAAGTTTGTACAAAAAAGCAG GCTGGGCCACCATGGTGAGCAAGGGCGAG3' and Reverse *attB2* 5'GGGGACCACTTTGTACAAGAAAGCTGGGTGTCTAGATCCGGTGGATCC3'. The forward primer was designed in frame with the recommended reading frame to generate C- or N-terminal fusions. In addition, the CDS was preceded by a Kosack consensus. The reverse primer was designed to omit the stop codon and to maintain the recommended frame for N- or C-terminal fusions by multisite Gateway cloning. The attB1-egfp-attB2 flanked PCR product was gel purified (Qiagen) and subsequently mixed with pDONR221 and subjected to a BP

reaction for 1 hr. at room temperature. The reaction mixture was subsequently diluted fivefold in dH_20 (Nuclease free water) and electroporated into TOP10 electrocompetent bacteria and subsequently grown in the presence of kanamycin. In order to generate the C-terminal epitope entry plasmids we amplified the mCherry coding sequence with the following primers containing a forward attB2 and reverse attB3 primers: attB2 forward 5'GGGGACAGCTTTCTTGTACAAAGTGGACATGGTGAGCAAGGGCGAGG3' and attB3 Reverse 5'GGGGACAGCTTTCTTGTACAAAGTGGACATGGTGAGCAAGGGCGAGG3'. We designed an in-frame stop codon in the reverse oligo. The attB2-mcherry-attB3 PCR product was gel purified and mixed with pDNORP2r-P3 and subjected to BP reaction as described above. The attB4-attB1 flanked zebrafish *bactin2* promoter entry clone (p5Ebactin2) was kindly provided by Chi-Bin Chein. A list of all 3' entry clones generated for this study can be accessed at: http://lawsonlab.umassmed.edu/GWEntryplasmids.htm.

Generation of Destination Vectors. To generate the multisite gateway pCS-based plasmid (*pCSDest2)*: pCSDest was digested with *PstI* and *XhoI* to remove the attR2 site and replaced with an attR3 site from pDESTR4-R3 (Invitrogen). The multisite tol2 vector (pDestTol2pA) containing an attR4-attR3 cassette was constructed by Clemens Grabher and kindly provided by Chi-Bin Chien. A list of all Destination vectors generated for this study can be accessed at: http://lawsonlab.umassmed.edu/GWDestplasmids.htm

Generation of Expression Constructs. To generate the pCS*egfpmcherry* construct we mixed pME*egfp3*, p3E*mcherry* and pCSDest2 at equal molar ratios and performed an LR reaction using LR clonase II plus (Invitrogen) and incubated the mixture at room temperature for 1 hr. To generate the pTol2bactin2:*egfpmcherry*; pDestTol2pA was mixed with pME*egfp3* and p3E*mcherry* at equal molar ratios and subjected to a multisite LR reaction using the LR clonase II plus (Invitrogen) recombinase. The reaction was incubated as described above. After incubation, the LR reactions were diluted fivefold in dH_2O and electroporated into TOP10 electrocompetent cells. The cells were subsequently selected in the presence of amplicillin. Clones were screened by PCR and/or mini prep (Qiagen). To construct pTol2HSP:vegfc-2Acherry we subjected pDestTol2pA, pME*vegfc-w/ostp*, p5EHSP70prm, and p3E-2Amcherry to a multisite LR reaction as described above. To construct pTol2HSP:dsRED-IRESnlsegfp we subjected pTol2DestpA:CG (Chi-Bin Chein), pMEdsRedEX, p5EHSP70prm (Chi-Bin Chein), and p3E-IRESnlsegfp (Chi-Bin Chein) to multisite LR reaction. To generate the pTol2*flieb:vegfc-2Amcherry* and pTol2*flieb:egfp-2Amcherry* we subjected pDestTol2pA, pME*vegfc-w/ostp* (for *vegfc* expression) or pME*egfp3* (*egfp* control expression), and p3E-2Amcherry to a multisite LR reaction as described above.

Injections

To generate mRNA for injection, pCS vectors were digested with *NotI,* extracted with phenol/chlorophorm and subsequently ethanol precipitated. Koichi Kawakami kindly provided the pCS2-TP plasmid encoding the Tol2 Transposase. The linearized plasmids were used in a standard *in vitro* transcription reaction using SP6 polymerase (Message Machine Ambion). We performed mRNA injections as described elsewhere[25]. For injections using Tol2 constructs, we injected one–cell stage embryos with 25pg of Tol2 *transposase* mRNA and 25pg of indicated Tol2-containing plasmid. For injections involving genetic knockdown, we used a previously published morpholino targeting the *flt4* transcript. At after injection with the indicated Tol2 and *transposase* constructs, embryos were separately a injected with either 2ng of Flt4 SD3 MO or Ctrl MO at the 4-8 cell stage[14].

Imaging and Phenotypic analysis

In order to monitor fluorescent protein expression and embryo morphology we made use of a Leica MZFLIII microscope equipped with epifluorescence. Digital images were captured using an AxioCam camera and Axiovision software. Confocal images were captured on a Leica SP2 confocal microscope. *hsp70*:*vegfc-2Acherry* and *hsp70*:*dsRED-IRESnlsegfp* injected Tg(*fli1a*:egfp)^{y1} embryos were heat shocked at 18 somite stage (ss) in egg water (ref). at 40℃ for 20 min. Embryos where allowed to develop normally at 28℃ and

subsequently analyzed for Vegfc-2AMcherry or DsredEx expression and ectopic segmental artery branching at 28 hpf. *flieb:vegfc-2Amcherry* and *flieb:egfp-2Amcherry* injected Tg(*fli1a:*egfp)^{y1} embryos were analyzed at 30 hpf for segmental arteries expressing Vegfc-2AMcherry or Egfp-2AMcherry, respectively.

Results

Multisite Gateway Cloning Technology

Gateway cloning technology is a universal system based on site-specific recombination reactions that allow rapid cloning of a gene of interest into multiple expression clones. This technology has been further engineered to allow simultaneous directional cloning of a promoter, gene of interest and epitope tag in to one expression clone; a method referred to as multisite Gateway cloning. A specific overview of this technology can be found in Appendix II.

Construction of a Gateway compatible cassette that allows expression of C-terminal tagged ORFs to analyze gene function in zebrafish.

Visually monitoring Vegfc expression in a spatial or temporal manner requires labeling it with a fluorescent epitope tag. Vegfc is a secreted factor that contains a signal sequence that allows it to be secreted. As a result, placing an N-terminal tag may affect is localization due to interference with the signal sequence, as observed in studies of membrane proteins. In these cases, placing an epitope tag on the C-terminus may avoid localization defects [137]. To construct a system that allowed for the rapid parallel construction and testing of C-terminus epitope tagged ORFs, we took advantage of multisite Gateway technology and engineered a Destination vector that allowed simultaneous directional cloning of an ORF fused to a 3' epitope tag.

Generally, Destination vectors contain attR1 and attR2 sites, which recombine with attL1 and attL2 of a given Middle entry clone(usually containing an ORF of interest). In this case we wanted to engineer a Destination vector that allowed introduction a second DNA fragment (3' epitope tag). As described in Appendix I, 3' entry clones (usually encoding an epitope tag) are flanked by attR2 and attL3 sites, thus allowing recombination of the attL2 site contained in the Middle entry clone and attR2 site contained in the 3'entry clone (Figure 1A, notice blue box recombine with orange boxes, attL2/attR2). Simultaneous and directional cloning of these fragments into a Destination vector would require the existence attR1 and attR3 sites in the Destination plasmid itself, allowing recombination of the remaining attL1 site of the Middle entry clone and attL3 site of the 3'entry clone (Figure 1A, notice, attL1/attR1 as well as attL3/attR3 recombination). The resulting clone is an expression vector containing a 3' epitope tagged ORF(Figure 1A). To generate such a Destination vector we modified the pCS vector, a commonly used vector to generate mRNA for gain-of-function analysis in zebrafish, so that in contained an attR1-attR3 cassette (Figure 1A, pCSDest2, See Methods).

As proof of principle we first used pCSDest2 to generate pCS*egfpmcherry*. To generate appropriate Entry clones for multisite Gateway recombination we constructed a 3' Entry clone encoding *mcherry* (p3Emcherry, see methods) (Figure 1B) and middle entry clone encoding *egfp*, minus the stop codon (pMEegfp3, see methods) (Figure 1B). We subsequently subjected pCSDest2,

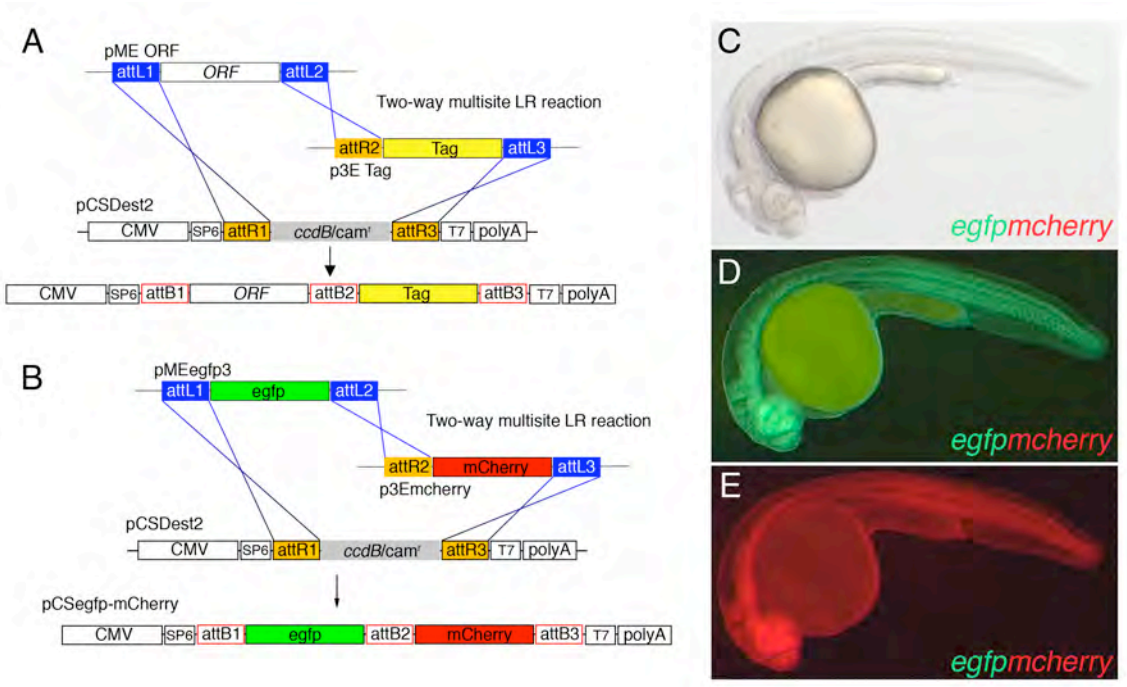

Figure 1. Appendix I Multisite Gateway approach to generate C-terminal fusion proteins in a pCS-based vector.

A. Depiction of the two-way multisite Gateway LR reaction between pMEORF, p3E Tag and pCSDest2 to generate C-terminal fusion proteins. Recombination can only occur between corresponding attL(Blue) and attR (Orange) sites resulting in the proper orientation in the Dest vector (pCSDest2), which contains an attR1 and attR3 site. This generates the pCSORF-Tag. **B.** Depiction of the two-way multisite Gateway LR reaction between pENTRegfp3, p3Emcherry and pCSDest2 to generate pCS*egfpmcherry*. Note that att sites are not to scale. **C-E**. A 24 hpf embryo injected with 100 pg of *egfp-mcherry* mRNA synthesized from the plasmid generated in B; lateral view, dorsal is up, anterior to the left; all pictures are from the same embryo. **C.** Transmitted light illumination. **D.** Illumination to visualize green fluorescence. **E:** Illumination to visualize red fluorescence.

pMEegfp3 and p3Emcherry to a multisite LR reaction and generated the pCS*egfpmcherry* construct (Figure 1B). Notice that corresponding attL and attR sites recombine (Figure 1B, Blue and Orange boxes recombine). The resulting pCS*egfpmcherry* vector was linearized and used to synthesized mRNA encoding the *egfpmcherry* fusion. Wild type embryos were injected at the one-cell stage with mRNA encoding *egfpmcherry* and subsequently monitored for transgene expression. We evidenced expression of EGFP-mCherry fusion in injected embryos at 24 hpf (Figure 1C-E). Taken together, these data demonstrate that the Gateway multisite system can be adapted for use with vectors commonly used to study gene function in zebrafish. Importantly, they demonstrate that this system can be used to tag ORFs at the C-terminus with a variety of epitope tags and test their functionality in a rapid manner.

Validation of Gateway compatible transgenic constructs for use in zebrafish.

Although ectopic expression of mRNA is often the easiest way to assess gene function in zebrafish, it often leads to unwanted developmental defects or indirect effects that can complicate a genetic analysis. Given the highly specific expression domains of certain genes, it is often preferable to control forced gene expression at the spatial and/or temporal level, which requires the application of transgenic DNA constructs containing the appropriate promoter elements. However, injection of transgenic DNA constructs in zebrafish results in high

mosiacism precluding the immediate analysis of the transgene, thus requiring higher doses of injection to achieve acceptable mosaic expression. Unfortunately, the increase of injection dose often results in toxic effects that decrease embryo viability. Recently, the development of the Tol2 transgenic vectors has alleviated the aforementioned drawbacks. The Tol2 transposon belongs to the hAT family of transposons. This transposon was originally isolated from the Japanese Medaka fish (*Oryzias latipes*) and has proved to be an efficient way to facilitate transgene integration into the zebrafish genome [138]. Integration of the Tol2 element is mediated by the Tol2 Transposase, which catalyzes the movement of the Tol2 transposon.

To generate a system that allowed spatial and/or temporal control over expression of a tagged Vegfc; we first tested an engineered Gateway compatible Tol2 transgenic vector[139] for its ability to robustly induce transient transgenesis in zebrafish embryos. This vector contains an attR4-attR3 cassette Gateway cassette that allows the incorporation a promoter fragment upstream of an ORF and its C-terminal tag (Figure 2A, referred to as pDestTol2pA)[139]. As proof of principle, we used pDestTol2pA to generate a construct that drove ubiquitous expression of the EgfpMcherry fusion protein(above). To do this we used a *bactin2* promoter entry plasmid (p5E*bactin2*) (Figure 2A); this promoter has been shown to robustly drive transgene expression following integration (personal communication). We performed a three-way multisite LR reaction using p5E-*bactin2*, pME*egfp3*, and p3E*mcherry* together with the pDestTol2pA plasmid

(Figure 2A, again notice Blue boxes recombine with Orange boxes). This created a plasmid in which the *bactin2* promoter was placed upstream of an *egfp-mcherry* fusion coding sequence (pTol2bactin2:*egfp-mcherry*) (Figure 2A). We subsequently co-injected this plasmid with *transposase* mRNA into one-cell stage wild type zebrafish embryos. At 26 hpf we noted that embryos injected with pTol2bactin2:*egfp-mcherry* displayed both green and red fluorescence in identical mosaic patterns (Figure 2B-D). These results indicate that it is possible to generate Tol2-based constructs in which the expression of C-terminally tagged fusion proteins are driven by a tissue-specific promoter in one single cloning step.

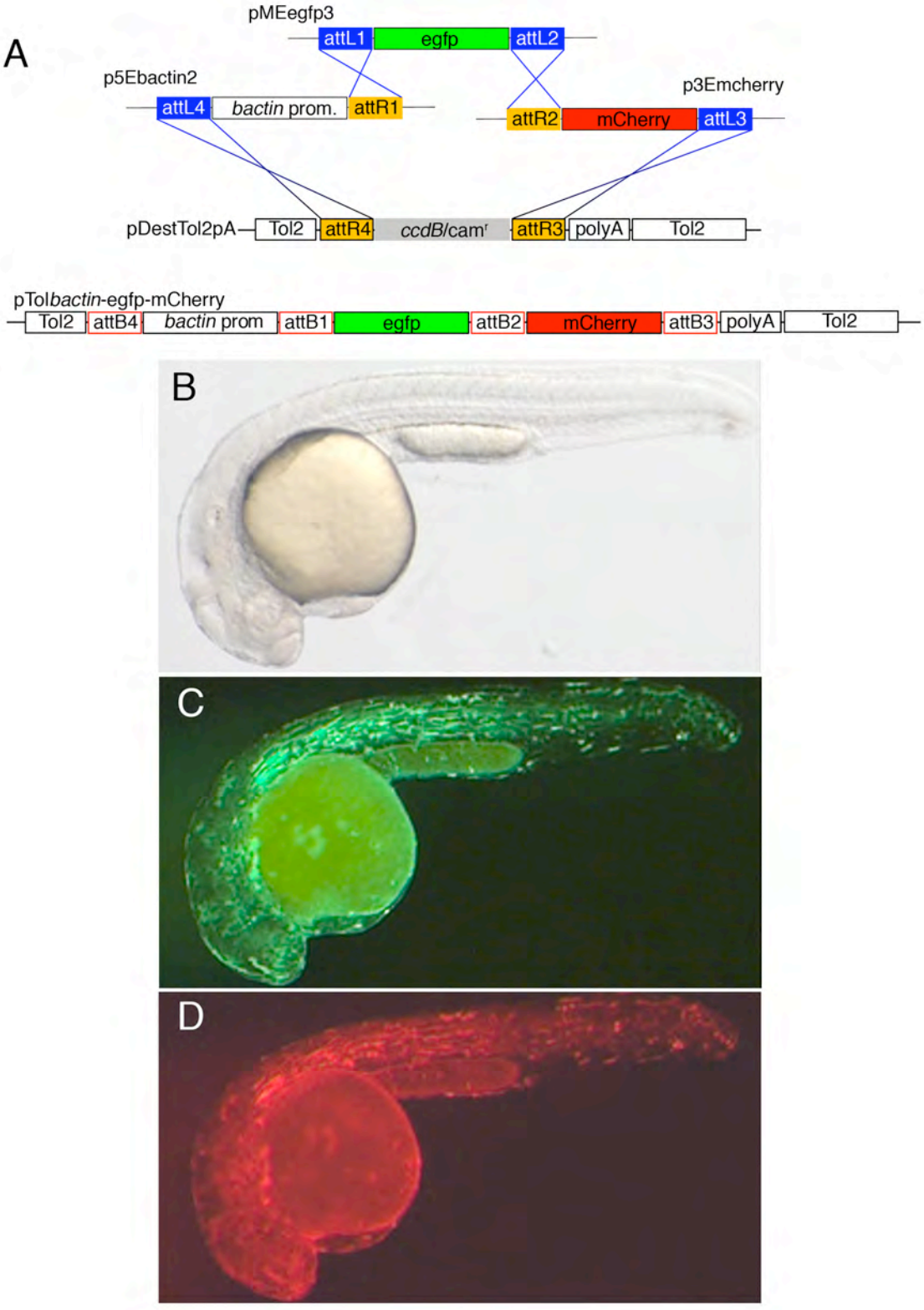
A
pMEegfp3
attL1
egfp
attL2
p5Ebactin2
attL4
bactin prom.
attR1
p3Emcherry
attR2
mCherry
attL3
pDestTol2pA
Tol2
attR4
ccdB/camr
attR3
polyA
Tol2
pTolbactin-egfp-mCherry
Tol2
attB4
bactin prom
attB1
egfp
attB2
mCherry
attB3
polyA
Tol2
B
C
D

Figure 2. Appendix I Multisite Gateway cloning to generate a tissue-specific Tol2 construct for expression of C-terminal fusion proteins.

A. Three-way multisite reaction into pDestTol2pA. The *egfp* and *mcherry* Entry clones are the same as those used in Figure 1. Note that att sites are not to scale. **B–G**. Embryos injected with 25 pg transposase mRNA and 25 pg pTol*bactin2:egfp-mcherry*; lateral view, dorsal is up, anterior to the left. **B.** Transmitted light illumination. **C.** Illumination to visualize green fluorescence. **D.** Illumination to visualize red fluorescence. E–G: Confocal micrographs; embryo is different than the one pictured in B. **E.** Mosaic green fluorescence of Egfp-mCherry observed in epidermal cells (arrows) and muscle cells (arrowheads). **F.** Red fluorescence of Egfp-mCherry. **G.** Overlay of images in E,F.

Spatial and Temporal over-expression of Vegfc reveals a conserved pro-angiogenic effect during developmental angiogenesis in zebrafish.

Until recently the effect of Vegfc on blood vessels during embryonic development was unknown [15]. To investigate whether there was a conserved role for Vegfc on blood vessel physiology in zebrafish, we induced expression of Vegfc in a temporal and endothelial cell-autonomous manner during embryonic development in zebrafish**.** To analyze the effects of Vegfc on blood vessels in temporal manner we used the Gateway multisite technology and Tol2 Gateway vectors to generate a heat-shock inducible Vegfc Tol2 transgenic vector (pTol2*hsp70:vegfc-2Acherry*) (Figure 3A). We used a fragment of the *hsp70* promoter that has previously been used for heat shock induction[139], to drive expression of vegfc. To visualize vegfc expressing cells, *vegfc* was fused in frame to *mcherry* separated by a viral 2A peptide sequence, which allows the production of multiple proteins from a single transgene[140]; this transgene is capable of rescue a *vegfc* mutant phenotype(Chapter III). We injected pTol2*hsp70:vegfc-2Acherry* together with tol2 *transposase* in to Tg(*fli1a:*egfp)^{y1} embryos. The Tg(*fli1a:*egfp)^{y1} is a transgenic zebrafish line allows visualization of endothelial cells which express Egfp. Injected embryos were subsequently heat-shocked at 18 hpf. At this time point embryos are just beginning to undergo angiogenesis from the DA to form SeA [7,28]. We noted that after heat shock, transiently injected *hsp70:vegfc-2Acherry* embryos express Mcherry in a ubiquitous mosaic pattern at 28 hpf. Mcherry expression was evidence of

presumed sites of transgene expression (Figure 3C, notice yellow arrowhead). Analysis of the blood vessels in these embryos showed ectopic branching of the developing SeA within the horizontal myseptum at 28 hpf (Figure 3B-C and F), while SegA in non heat-shocked embryos developed normally (personal observation). By contrast, heat shocked *hsp70:dsRedEx-IRESnlsegfp* embryos displayed normal segmental arteries (Figure 3D-F). In this case, the 3' epitope included an Internal Ribosomal Entry Sequence (IRES) that allowed bicistronic mRNA production of *dsRedEx* and *nls-egfp*. Together these data suggest that early forced expression Vegfc during blood vessel development is able to induce angiogenic effects on endothelial cells.

It is very well established the Vegfc functions by binding and inducing phosphorylation of its corresponding receptor, Flt4 [58,60]. To investigate whether the effects of Vegfc on blood vessels were through Flt4 during developmental angiogenesis in zebrafish, we genetically down regulated *flt4*, using an antisense morpholino (MO), in heat shocked *hsp70*:*vegfc-2Amcherry* injected embryos. We observed that heat shock induced expression of *vegfc* failed to induce ectopic angiogenic sprouting in *flt4* deficient embryos when compared to heat shocked *hsp70*:*vegfc-2Amcherry* control morphant embryos (Figure 3G-K). Taken together, these data suggest that the effect of Vegfc on blood vessels *in vivo* is mediated through its receptor Flt4.

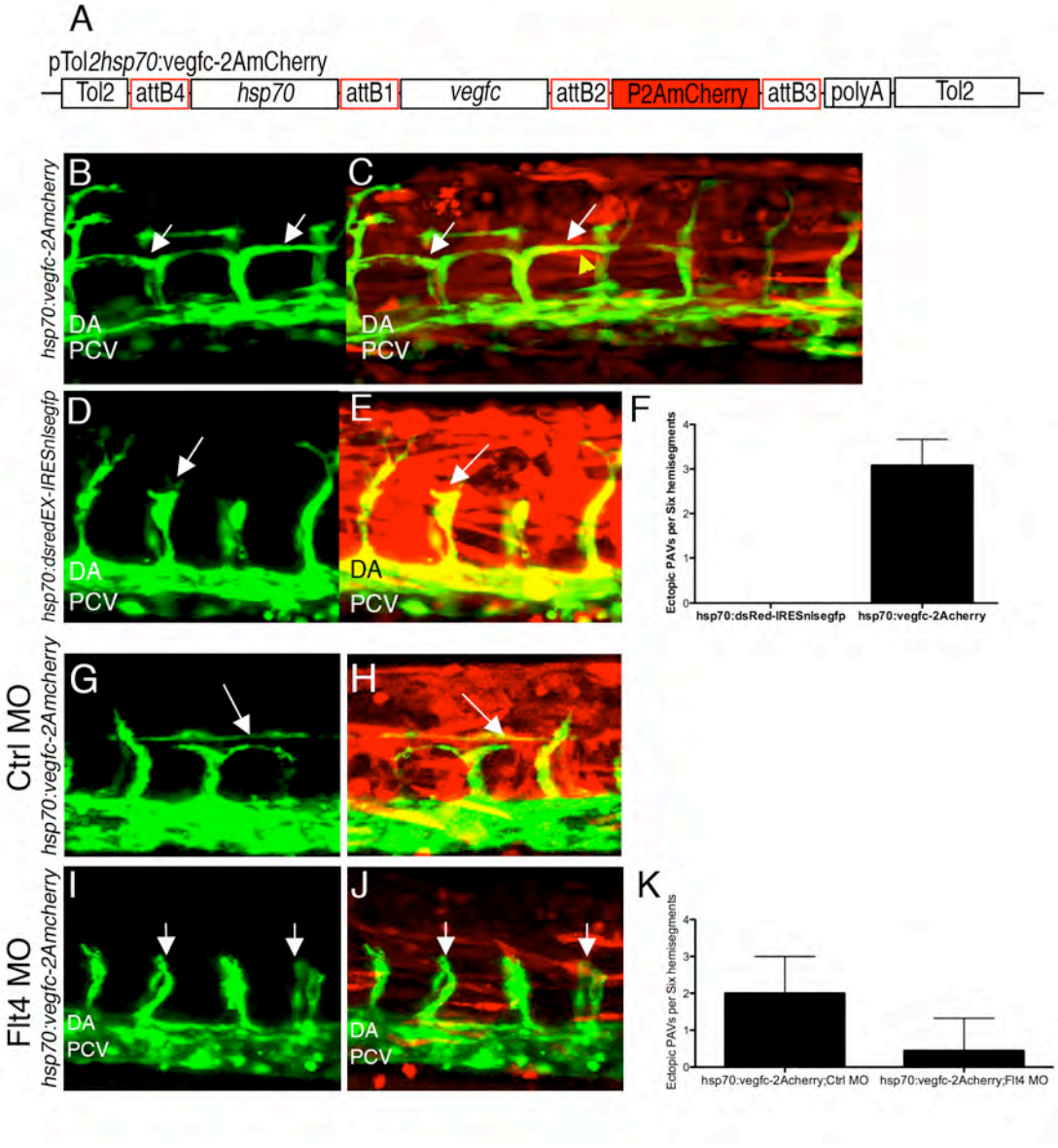
A
pTol2hsp70:vegfc-2AmCherry
Tol2
attB4
hsp70
attB1
vegfc
attB2
P2AmCherry
attB3
polyA
Tol2
B
C
hsp70:vegfc-2Amcherry
DA
PCV
D
E
hsp70:dsredEX-IRESnlsegfp
F
Ectopic PAVs per Six hemisegments
hsp70:dsRed-IRESnlsegfp
hsp70:vegfc-2Acherry
Ctrl MO
G
H
I
J
Flt4 MO
K
hsp70:vegfc-2Acherry;Ctrl MO
hsp70:vegfc-2Acherry;Flt4 MO

Figure 3. Appendix I Temporally controlled pro-angiogenic effects of Vegfc on blood vessels during embryonic blood vessel development are dependent on *flt4*.

A. Heat-shock inducible Vegfc Tol2 transgenic vector, pTol2*hsp70:vegfc-2Acherry*, generated by Multisite Gateway cloning **B-E and G-J.** Confocal micrographs of trunk blood vessels at 28 hpf. Anterior is to the left, dorsal is up. DA, Dorsal Aorta. PCV, Posterior Cardinal Vein. **(B-C)** Tg(*fli1a:*egfp)^{y1} embryos injected with 25 pg transposase mRNA and 25 pg pTol2*hsp70:vegfc-2Acherry* and heat shocked at 18 hpf. C. Yellow arrowhead indicates vegfc transgene expression. B-C. White arrows indicate ectopic branching of SegA. **(D-E)** Tg(*fli1a:*egfp)^{y1} embryos separately injected with 25 pg transposase mRNA and 25 pg pTol2*hsp70:dsRedEx-IRESnlsegfp* and heat shocked at 18 hpf. White arrows indicate normal SeA. **(G-H)** Tg(*fli1a:*egfp)^{y1} embryos injected with 25 pg transposase mRNA and 25 pg pTol2*hsp70:vegfc-2Acherry* and subsequently with Ctrl MO. Embryos were heat shocked at 18 hpf and analyzed at 28 hpf. G-H. White Indicate ectopic branching of SeA. **(I-J)** Tg(*fli1a:*egfp)^{y1} embryos injected with 25 pg transposase mRNA and 25 pg pTol2*hsp70:vegfc-2Acherry* and subsequently with *flt4* MO. Embryos were heat shocked at 18 hpf and analyzed at 28 hpf. White arrows indicate normal SeA. **F and K.** Quantification of ectopic SegA branching per six hemisegments for the indicated manipulation. Values shown are based on the average of three independent experiments.

Vegfc expression is restricted to endothelial cells lining the DA and SeA in during development in zebrafish (personal observation)[14]. Recent evidence in mouse shows that *vegfc* induces angiogenesis when expressed in the endothelium during embryonic development [84]. To investigate whether Vegfc had a similar conserved role in zebrafish, we again used the Gateway system and Tol2 Gateway vectors to generate an endothelial driven Vegfc construct. In this case we used an Ets-binding element from the *fli1a* gene (referred to hereafter as *fli1ebs*) to drive expression of Vegfc (pTol2*fliebs:vegfc-2Amcherry*). As above, *vegfc* was fused to *2Amcherry* to visualize expression. We noted that Tg(*fli1a*:egfp)^{y1} embryos transiently expressing *fli1ebs:vegfc-2Amcherry* in developing segmental arteries induced ectopic branching of segmental arteries within the horizontal myoseptum, as compared to transient expression of *egfp-2Amcherry,* which had no effect (Figure 4A-C). The ectopic branching of segmental arteries was similar to those observed during temporal missexpression of Vegfc (above). Taken together, these data suggest a pro-angiogenic role for Vegfc during embryonic blood vessel development in zebrafish.

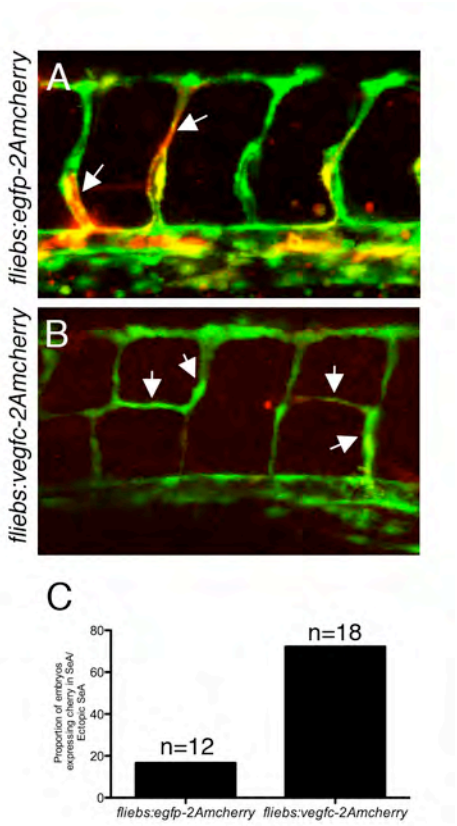
A
fliebs:egfp-2Amcherry
B
fliebs:vegfc-2Amcherry
C
Proportion of embryos expressing cherry in SeA/ Ectopic SeA
80
60
40
20
0
n=12
n=18
fliebs:egfp-2Amcherry
fliebs:vegfc-2Amcherry

Figure 4. Appendix I Pro-angiogenic effects of cell autonomous expression of Vegfc during blood vessel formation in zebrafish.

(A-B) Confocal micrographs of trunk blood vessels at 30 hpf. Anterior is to the left, dorsal is up. DA, Dorsal Aorta. PCV, Posterior Cardinal Vein. **A.** Tg(*fli1a:*egfp)^{y1} embryos injected with 25 pg transposase mRNA and 25 pg pTol2*fliebs:egfp-2Acherry*. White arrows indicate co-localization of transgene expression in SegA. **B.** Tg(*fli1a:*egfp)^{y1} embryos injected with 25 pg transposase mRNA and 25 pg pTol2*fliebs:vegfc-2Acherry*. White arrows indicate co-localization of the transgene in SegA that display ectopic branching. **C.** Quantification of ectopic branching of SegA in for *fliebs:egfp-2Acherry* (n=12) and *fliebs:vegfc-2Acherry* (n=18) injected embryos expressing the transgene in SeA.

Discussion

In this study, we show that spatial and temporal overexpression of Vegfc during developmental angiogenesis induces pro-angiogenic effects in zebrafish. To engineer a system that allows spatial and temporal control of Vegfc, we first adapted and modified Gateway cloning cassettes into vectors commonly used to assess gene function in zebrafish and subsequently validated the ability to rapidly generate vectors that allow specific expression of a 3' tagged ORF. We generate constructs that allow spatial and temporal control of Vegfc during blood vessel development and find that Vegfc has conserved pro-angiogenic effect on blood vessels when it is temporally missexpressed or expressed in an endothelial-cell autonomous manner. Moreover, we find that the pro-angiogenic effects of Vegfc are likely through Flt4.

Our study shows that the Gateway cloning system is modular and can be used to establish a collection of appropriate vectors that are suitable for transient and transgenic functional studies in zebrafish. Projects similar to the one described here have generated extensive Gateway compatible gene collections for human, *C. elegans*, *Schizosaccharomyces pombe* and viral genes [141-144]. We illustrate the combined use of multisite Gateway cloning and a collection of zebrafish compatible vectors to rapidly clone C-terminally tagged ORFs and promoter driven C-terminal tagged ORFs. One important advantage of this method of cloning is that it obviates the use of cloning strategies using restriction enzymes

and ligases. In addition, it creates a standardized cloning system where clones are sequence verified once, and can then be used in a modular fashion to generate multiple tagged versions of an ORF. As a consequence, this system results in more time and effort directed toward functional analysis of genes rather than toward plasmid construction.

Once Entry clones were inserted into Tol2 Gateway compatible Destination vectors the Entry sequences were flanked on either side by a*tt*B sites, which could presumably affect protein function or localization. However, during the course of our study we did not observe any overt defects in protein function or localization. This seems to be the case for the majority ORF analyzed (personal observation) and is consistent with other reports, which did not observe ectopic expression [145,146]. The Tol2 vectors contain *cis* elements that facilitate integration into the genome. Although the LR recombinases have been shown to be specific, it is possible that this recombinase could interfere with the Tol elements flanking the transgenic plasmid. However, we did not observe any evidence of this scenario in our multisite cloning reactions, implying that the LR clonase is highly specific and does not interfere with the Tol2 elements of the transgenic vector.

Previous studies in zebrafish showed that exogenous *vegfc* mRNA induced mild angiogenic effects on blood vessels during embryonic development[15]. Given the temporal and restricted expression domain of *vegfc* we a decided to investigate the effect of Vegfc on blood vessels during developmental angiogenesis using

promoter elements to restrict transgene expression. Here we show that temporal exogenous expression of *vegfc*, using heat shock promoter, is strongly angiogenic during developmental angiogenesis. Temporal misexpression of Vegfc at 18-24 hpf coincides with sites of active angiogenesis as well as the concomitant expression of Vegfrs such as *flt4* and zebrafish homologue of *vegfr2* (*kdrl*). Given that Vegfc has the ability to activate Flt4 and Kdrl[60], these data imply that Vegfc is able to elicit an angiogenic response through Vegfrs.

In particular, we found that *flt4* but not *kdrl* (personal observation) both of which are expressed is segmental arteries during blood vessel development, is required in order for Vegfc to prompt a pro-angiogenic phenotype. These data suggest that Vegfc specifically acts through Flt4 to induce the observed ectopic vessel sprouting. Consistent with this idea, we find that forced expression of Vegfc in segmental arteries also induces ectopic branching of blood vessels, further implying a role for Vegfc through Flt4 during this process; given the co-expression of both these factors in SeA, it is probably doing so in an autocrine mode of signaling. Together these data imply a conserved pro-angiogenic effect by Vegfc/Flt4 signaling on blood vessels in zebrafish.

In conclusion, this work describes a cloning system that allows for rapid construction of transgenic vectors to analyze gene function in zebrafish. Using this cloning technique we generated a system that allows spatial and temporal expression of Vegfc in order to determine if it had angiogenic effect on blood vessels during embryonic development. We find that Vegfc likely can act in a cell

autonomous manner and drives angiogenic effects through Flt4. The findings should prompt new investigation into the functional and cell autonomous role of Vegfc during developmental angiogenesis.

APPENDIX II

Overview of Multisite Gateway Technology

Gateway cloning technology is based on site-specific recombination reactions that involve *cis* elements and the λ integrase family of recombinases[147]. Importantly, these recombinases are conservative and highly specific, catalyzing a reaction that yields no net gain or loss of nucleotides. The *cis* elements are homologous in nature and are designated *att* sites. The multisite Gateway system (Invitrogen) makes use of several engineered *att* sites that recombine in a specific and directional manner to clone up to five fragments of DNA. In this study, we will make use of the single-insert and three-insert multisite Gateway system.

The “BP” recombination reaction describes the recombination between *attB* and *attP* sites on two different DNA fragments. (Figure 1A). Typically, this reaction is used to recombine a fragment generated by Polymerase Chain Reaction (PCR), which is flanked by *attB* sites, into a surrogate vector containing *attP* sites. This surrogate vector is usually referred to as a DONOR vector (Figure 1A, pDONR221). This reaction is catalyzed by bacteriophage λ intergrase (Int) and integration host factor (IHF). Together, these factors are referred to as “BP clonase” (Invitrogen). BP clonase catalyzes recombination of two DNA fragments

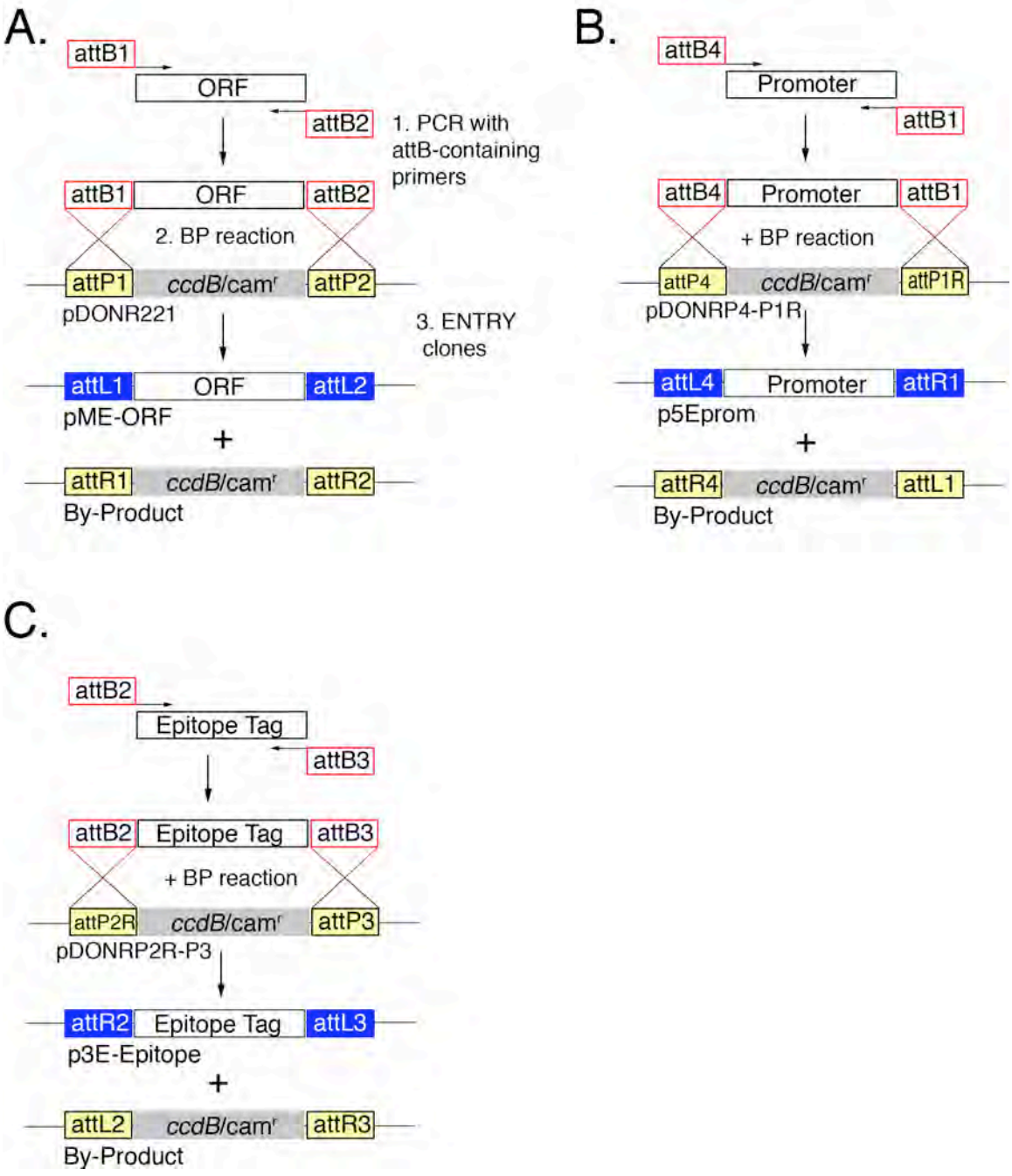
A.
attB1
ORF
attB2
1. PCR with attB-containing primers
attB1
ORF
attB2
2. BP reaction
attP1
ccdB/camr
attP2
pDONR221
3. ENTRY clones
attL1
ORF
attL2
pME-ORF
+
attR1
ccdB/camr
attR2
By-Product
B.
attB4
Promoter
attB1
attB4
Promoter
attB1
+ BP reaction
attP4
ccdB/camr
attP1R
pDONRP4-P1R
attL4
Promoter
attR1
p5Eprom
+
attR4
ccdB/camr
attL1
By-Product
C.
attB2
Epitope Tag
attB3
attB2
Epitope Tag
attB3
+ BP reaction
attP2R
ccdB/camr
attP3
pDONRP2R-P3
attR2
Epitope Tag
attL3
p3E-Epitope
+
attL2
ccdB/camr
attR3
By-Product

Figure 1. Appendix II

Gateway BP recombination reactions. **A:** Depiction of a BP reaction for a polymerase chain reaction amplified open reading frame. **B:** Depiction of a BP reaction for a polymerase chain reaction amplified promoter fragment, notice the attB4 and attB1 sites. Depiction of a BP reaction for a polymerase chain reaction amplified epitope tag. Notice the attB1 and attB3. Note that att sites are not to scale.

that yields two plasmids or DNA fragments that now correspond to *att*L and *att*R sites (Figure 1A). The *att*L site containing plasmid forms part of what is now called a “Middle Entry” plasmid (pME-ORF). Typically, these Middle Entry clones encode a reporter or the open reading frame (ORF) for a gene of interest. To promote high efficiency recovery of Entry plasmids, the Gateway system relies on a counter-selection method. In this case, DONOR vectors contain a *ccd*B cassette that is toxic to standard bacterial strains used for cloning (e.g., DH10B, DH5α); an adjacent chloramphenicol resistance gene allows maintenance of the cassette in *ccd*B-tolerant cells. Therefore, un-recombined DONOR vector, or attR-containing byproducts will not be propagated after a BP reaction resulting in high efficiency recovery of the desired Entry clone.

As described earlier, it is often necessary to use a promoter fragment to drive specific expression of a reporter or gene of interest. To generate these clones, promoter fragments can be amplified with flanking *att*B4-*att*B1sites and subsequently inserted into a surrogate vector containing engineered *att*P4 and *att*P1R sites (pDONRP4-P1R) performing a BP reaction (Figure 1B). Similar to the BP reaction described above, the reaction yields fragments of DNA that correspond to *att*L4-*att*R1 and *att*R4-*att*L1 sites. The plasmid containing the *att*L4-*att*R1 site is referred to as a “5’ Entry plasmid” (p5E-Prom). The *att*R4-*att*L1 fragment contains the *ccd*B/chloramphenicol cassette and is counter selected (Figure 1B). Additionally, a BP reaction can be used to generate “3’ Entry plasmid” (p3E-Epitope) (Figure 1C). These clones often contain C-terminal

epitopes encoding fluorescent proteins such as Mcherry. In this case, BP clonase recombines pDONRP2R-P3, which is flanked by *att*P2R and *att*P3 sequences, and an amplified PCR product flanked by *att*B2 and *att*B3 sequences (Figure 1C). This recombination event results in an *att*R2-*att*L3 flanked p3Entry plasmid and counter a selected *ccd*B/chloramphenicol fragment flanked by *att*L2-*att*R3 sequences. Together, these three forms of Entry plasmids serve as building blocks that can be used to simultaneously generate multiple variants of a particular expression clone in a rapid manner.

Once appropriate Entry plasmids are generated, they can be used in a multisite LR reaction with corresponding "Destination" vectors which contain homologous *att*R sites. Usually, Destination vectors contain elements for eukaryotic or prokaryotic expression (Figure 2A, ex. CMV, SP6). Int, IHF, and excisionase (referred to as LR clonase) catalyzes the LR recombination. The LR reaction results in the recombination of corresponding *att*L and *att*R sites contained in the Entry and Destination plasmids (Figure 2A). The resulting plasmid is usually an Expression clone where the inserts are flanked by *att*B sites (Figure 2B, Blue boxes). In addition to this plasmid, a by-product plasmid that contains a ccdB/chloramphenicol cassette flanked by *att*P sites is also generated (Figure 2B, Orange boxes). As with the BP reaction, both un-recombined Destination vector and the *att*P-containing by-product are selected against by the presence of the *ccdB* gene in an LR reaction. Additionally, Destination vectors contain ampicillin resistance, while DONOR or Entry plasmids contain kanamycin

resistance cassettes. Thus, introduction of an LR reaction into standard cloning bacteria and selection on ampicillin results in high efficiency recovery of the desired Expression clone. The resulting Expression clone that contains the *att*B-flanked inserts can then be used for appropriate functional studies.

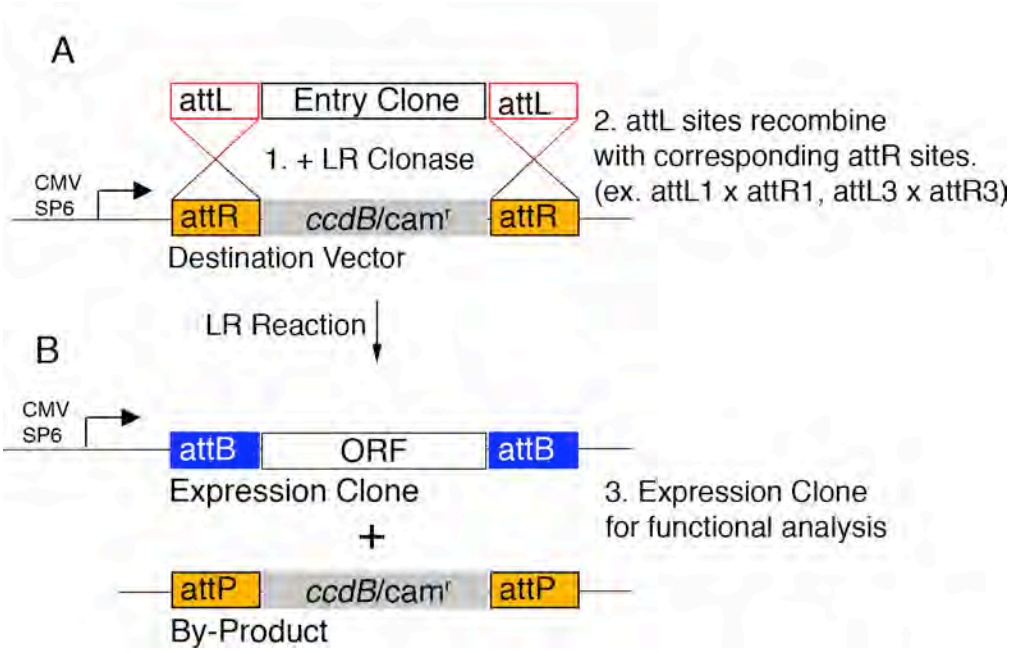

Figure 2. Appendix II

Gateway LR recombination reaction. **A.** Depiction of LR reaction between an ENTRY clone and a Destination vector. Note that att sites are not to scale. **B.** Depiction of Expression clone and attP site containing by-product.

APPENDIX III

in vivo forced expression of *vegfc^{um18}* induces ectopic branching of SeA

During the course of our biochemical characterization of Vegfc^{um18} in mammalian cells we noted that overexpression of VEGFC-ΔC (human equivalent of Vegfc^{um18}) led to its eventual secretion at 72 hours post transfection (Chapter 4 Figure 2C). To investigate whether overexpression of Vegfc^{um18} led its eventual secretion *in vivo*, we transiently overexpressed either *vegfc* or *vegfc^{um18}* in Tg(fli1a;egfp)y1 embryos using a heat shock inducible promoter. This promoter allows temporal control as well as high induction of transgene expression. To visualize ligand-expressing cells, we again fused 2Amcherry to both *vegfc* and *vegfc^{um18}*. In contrast to heat shocked hsp70a:*dsredEX-IRESnlsegfp* controls, we observed ectopic branching of segmental arteries at 26 hpf in hsp70a:*vegfc-2Amcherry* injected embryos heat-shocked at 18hpf (Figure 1A-D,G). Similarly, we noted that heat-shock overexpression of hsp70a:*vegfc^{um18}-2Amcherry* elicited ectopic branching of segmental arteries similar to those induced by hsp70a:*vegfc-2Amcherry* (Figure 1E-F). Consistent with our previous biochemical observations these data suggest that higher levels of Vegfc^{um18} expression result its eventual secretion.

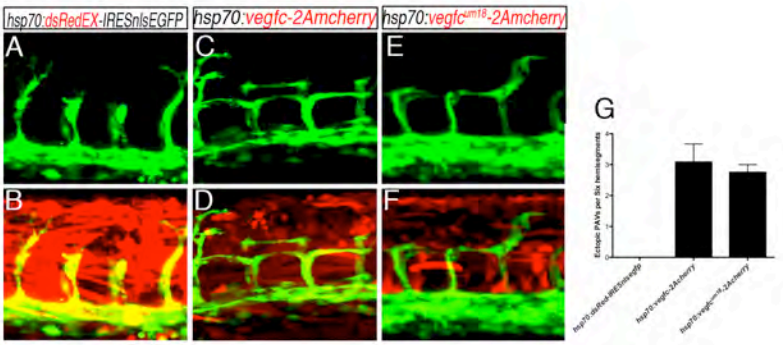
hsp70:dsRedEX-IRESnlsEGFP
hsp70:vegfc-2Amcherry
hsp70:vegfc^um18-2Amcherry
A
B
C
D
E
F
G

Figure 1. Appendix III Elevated misexpression of *vegfc*^{um18} induces pro-angiogenic effects on blood vessels.

(A-F). Confocal micrographs of trunk blood vessels at 28 hpf. Anterior is to the left, dorsal is up. DA, Dorsal Aorta. PCV, Posterior Cardinal Vein. Red channel indicates sites transgene expression **(A-B)** Tg(*fli1a:*egfp)^{y1} embryos injected with 25 pg transposase mRNA and 25 pg pTol2*hsp70:dsRedEx-IRESnlsegfp*. Embryos were heat shocked at 18 hpf. **(C-D)** Tg(*fli1a:*egfp)^{y1} embryos injected with 25 pg transposase mRNA and 25 pg pTol2*hsp70:vegfc-2Acherry*. Embryos were heat shocked at 18 hpf. **(E-F)** Tg(*fli1a:*egfp)^{y1} embryos injected with 25 pg transposase mRNA and 25 pg pTol2*hsp70:vegfc*^{um18}*-2Acherry*. Embryos were heat shocked at 18 hpf. **(G)** Quantification of ectopic SegA branching per six hemisegments for the indicated manipulation. Values shown are based on the average of three independent experiments.

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